EBV prevents cAMP-mediated inhibition of DNA damage-induced apoptosis

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Aknowledgements

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Oslo, september 2006

Silje Rebekka Olsen

Summary

DNA damage responses are the basis for conventional cancer treatments like γ-irradiation and chemotherapy, and the p53 protein is a key mediator in this process. Our lab has recently demonstrated that elevation of intracellular cAMP also leads to inhibition of DNA damage-induced apoptosis, and results so far have shown that this inhibition is mediated by downregulation of p53 at the level of protein stabilisation (Hallan Naderi et al., manuscript in preparation).

Epstein-Barr virus is a DNA tumour virus implicated in the development of mononucleosis and cancers like Burkitt's lymphoma, Hodgkin's lymphoma, gastric cancer, and nasopharyngeal carcinoma. We have recently shown in our lab that EBV-infection of B-cells inhibits the antiproliferative effect of cAMP (Kloster et al., manuscript in preparation).

The aims of the present project were to unravel whether EBV-infection of B-cells prevents cAMP-mediated inhibition of DNA damage-induced apoptosis triggered by γ-radiation, and if so, to establish the mechanisms involved. Our experiments showed that EBV-transformed B-cells were more resistant to γ-radiation, and that EBV-infection indeed prevents cAMP-mediated inhibition of apoptosis induced by ionizing radiation. Thus, we revealed that the stability of p53 in EBV-infected B cells was largely unaffected by cAMP, whereas the stability of p53 in control cells was reduced. Our analysis of the mechanisms involved in the ability of EBV to prevent cAMP-mediated destabilization of p53 revealed that activation of Chk1 and Chk2 were unaffected, suggesting that the inhibitory effect of EBV on cAMP-mediated regulation of DNA damage-induced apoptosis is not mediated via checkpoint-control upstream of p53, but rather that the cAMP-mediated pathway as such is affected.

Abbreviations

AIDS	Aquired immune deficiency syndrome
AMP	Adenosine monophosphate
APAF-1	Apoptotic protease activationg factor-1
APS	Ammonium persulfate
A-T	Ataxia-teleangiectasia
ATM	Ataxia teleangiectasia mutated
ATP	Adenosine triphosphate
BART	Non-polyadenylated (non-coding) RNA
BCR	B-cell receptor
BHRF1	Bam HI fragment H rightward open reading frame 1
BID	BH3-interacting-domain death agonist
Bis	N,N-methylenebisacrylamide
cAMP	Cyclic adenosine monophosphate
CAK	Cdk-activating kinase
Cdk	Cyclin dependent kinase
Chk2	Checkpoint kinase 2
CKI	Cyclin dependent kinase inhibitor
COP-1	Coat protein complex 1
dH2O	Distilled water
EBER	Non-polyadenylated (non-coding) RNA
EBNA	EBV-nuclear antigen
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra acetic acid
Epac1 & 2	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorter
FADD	FAS-associated death domain
FBS	Fetal bovine serum
FDC	Follicular dendritic cell
GADD45	Growth arrest and DNA damage 45
HAUSP	Herpes virus associated ubiquitin-specific protease
HD	Hodgkins lymphoma
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HRS	Reed-Sternberg cells
Ig	Immunoglobulin
IM	Infectious mononucleosis
INK4	Inhibitor of Cdk4
LCL	Latently infected lymphoblastoid cell line
LMP	Latent membrane protein
MDM2	Murine double minute 2
MOMP	Mitochondrial outer membrane permeabilization

PBS	Phosphate buffered saline solution
PCNA	Proliferating cell nuclear antigen
PDA	Phosphodiesterase
PI	Propidium iodide
PKA	cAMP-dependent protein kinase
pRB	Retinoblastoma protein
RB	Retinoblastoma gene
RB2	Retinoblastoma-like 2
PTLD	Post-transplant lymphoproliferative disease
rNTP	Ribonucleoside triphosphate
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SMH	Somatic hypermutation
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor-1
TRADD	TNFR1-associated death domain
UV	Ultra violet
V	Volt
8-CPT-cAMP	8- (4- Chlorophenylthio) adenosine- 3', 5'- cAMP

CONTENTS:

1. INTRODUCTION

1.1 The Cell cycle

A cell reproduces by performing an orderly sequence of events in which it duplicates its contents and then divides into two daughter cells. This cycle of duplication and division, called the cell cycle, is the essential mechanism by which all living organisms reproduce. The details of the cell cycle vary from organism to organism and at different times in the organism's life. However, certain characteristics are universal. The cell cycle is defined as the period between two cell divisions, and the eukaryotic cell cycle is traditionally divided into four sequential phases: G1, S, G2 and M. Chromosome duplication occurs during S-phase of the cycle, whereas most other cell components are duplicated continuously throughout the cycle. The S- phase usually requires 10-12 hours, and occupies about half of the cell cycle in mammalian cells. In M-phase the duplicated chromosomes are segregated into individual nuclei (mitosis), and the cell splits in two (cytokinesis). The M-phase requires much less time, estimated to be less than one hour in mammalian cells. The S-phase and M-phase are usually separated by gap phases, G1 and G2. In this way the cell is able to grow as well as monitor the internal and external environment to ensure that conditions are suitable and that preparations are complete before the cell commits itself to S-phase and mitosis. G1 is particularly important in this respect. G0 (zero) is a resting state in which the cell can remain in for days, weeks or even years before resuming proliferation.

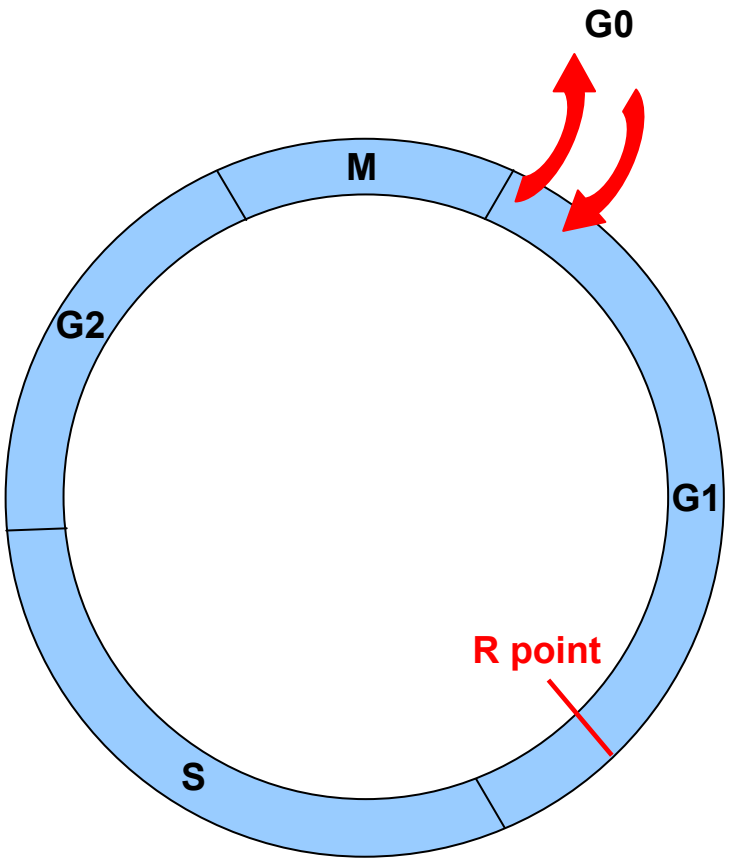


Figure 1: The cell cycle.

1.1.1 Cell cycle machinery

There are two key components in the cell cycle machinery; cyclins and cyclin-dependent kinases (Cdks). If the Cdks are not tightly bound to a cyclin in a cyclin-Cdk complex they have no protein kinase activity. Cdk levels are constant throughout the cell cycle, while cyclin levels undergo a cycle of synthesis and degradation. Therefore, cyclical changes in cyclin levels result in the cyclic assembly and activation of the cyclin-Cdk complex, which again trigger cell cycle events. Expressions of cyclins A and B are regulated periodically, whereas expression of cyclin D, and to a certain extent, cyclin E, is induced by mitogen stimulation.
There are four classes of cyclins. G1/S cyclins (cyclin E) binds Cdk2 at the end of G1 to commit the cell to DNA replication, S cyclins (cyclin A), binds Cdk2 during S-phase and are required for initiation of DNA replication. M cyclins (cyclin B), bind to Cdk1 to promote the events of mitosis. In most cells, a fourth class of cyclins, G1 cyclins (Cyclin D1, D2 and D3), helps promote passage through the restriction point (R) in late G1 by binding to Cdk 4 or 6.
(For review see Murray, A.W. 2004)

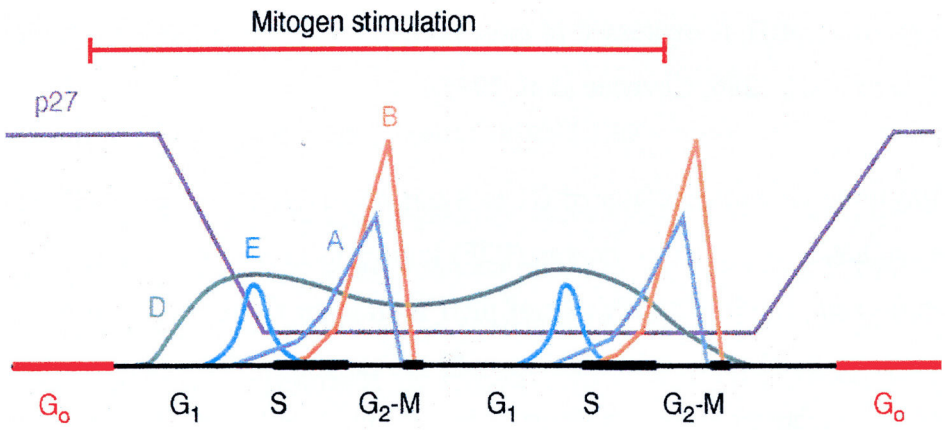


Figure 2: The fluctuation of cyclins and p27$^{(Kip1)}$ during the cell cycle. Cyclin E, A and B is expressed periodically, whereas D-type cyclins are expressed in response to mitogen stimulation. p27$^{(Kip1)}$ levels are high in quiescent cells, fall in response to mitogen stimulation and remain low in proliferating cells (Sherr, 1996).

In absence of cyclin, the active site in the Cdk protein is blocked by a slab of protein. Cyclin binding causes this slab to move away, resulting in only part activation of the Cdk enzyme. Full activation occurs when a Cdk-activating kinase (CAK) phosphorylates an amino acid near the entrance of the active site, causing a small conformational change that further increase the Cdk activity. The activity of the cyclin-Cdk complex can be inhibited by phosphorylation at a pair of amino acids in the roof of the active site by a protein kinase known as Wee1, while dephosphorylation of these sites by a phosphatase known as cdc25 increases the activity. Cyclin-Cdk complexes can also be regulated by the binding of Cdk inhibitory proteins (CKIs) (for review see (Sherr & Roberts 1999)). These act primarily in the control of G1- and S-phase. There are two distinct families of CKIs; the INK4 (inhibitor of Cdk4) family of proteins and the CIP/KIP family.

The INK4 family includes $p16^{(INK4a)}$, $p15^{(INK4b)}$, $p18^{(INK4c)}$ and $p19^{(INK4d)}$ and inhibit the CDK4/6-cyclinD complexes. The Cip/Kip family includes $p21^{(Cip1)}$, $p27^{(Kip1)}$ and $p57^{(Kip2)}$ that primarily interact with cyclin-Cdk2 complexes. The G1- phase of the cell cycle is the most regulated phase (Boonstra 2003; Deshpande et al.), since it contains the R point. This point is located at the end of the G1-phase, where the cell has to decide whether to proceed through the cell cycle and undergo cell division, or to exit the cycle and go to G0 (Pardee 1974). The control of G1- to S-phase progression is often disrupted in cancer, leading to unrestrained cell-cycle entry and cell proliferation.

1.1.2 The regulation of the R-point (G1-to S-phase transition): The Rb pathway

The best understood effects of G1-Cdk activity in animal cells are mediated by a gene regulatory protein called E2F. It binds to specific DNA sequences in the promoters of many genes that encode proteins required for S-phase entry. E2F function is controlled primarily by an interaction with the retinoblastoma protein (pRb), an inhibitor of cell cycle progression. The retinoblastoma gene (Rb) was the first tumour suppressor to be identified. The Rb gene product, pRb, is thought to be one of the most important proteins in G1/S-phase transition, as it has a crucial role in the control of the R point in G1. During G1, pRb binds to E2F and blocks the transcription of S-phase genes. In quiescent cells (G0), pRb is unphosphorylated. In cycling cells it has two phosphorylation forms; hypophosphorylated and hyperphosphorylated (Ezhevsky et al. 2001). pRb is sequentially phosphorylated throughout the cell-cycle by several cyclin-Cdk complexes. This is also terminated 'the Rb pathway'. When cells are stimulated by extracellular growth signals, active cyclinD-Cdk4/6 and later cyclin E-Cdk2 accumulates and phosphorylates Rb, sequentially reducing its affinity for E2F, allowing E2F to activate S-phase gene expression.

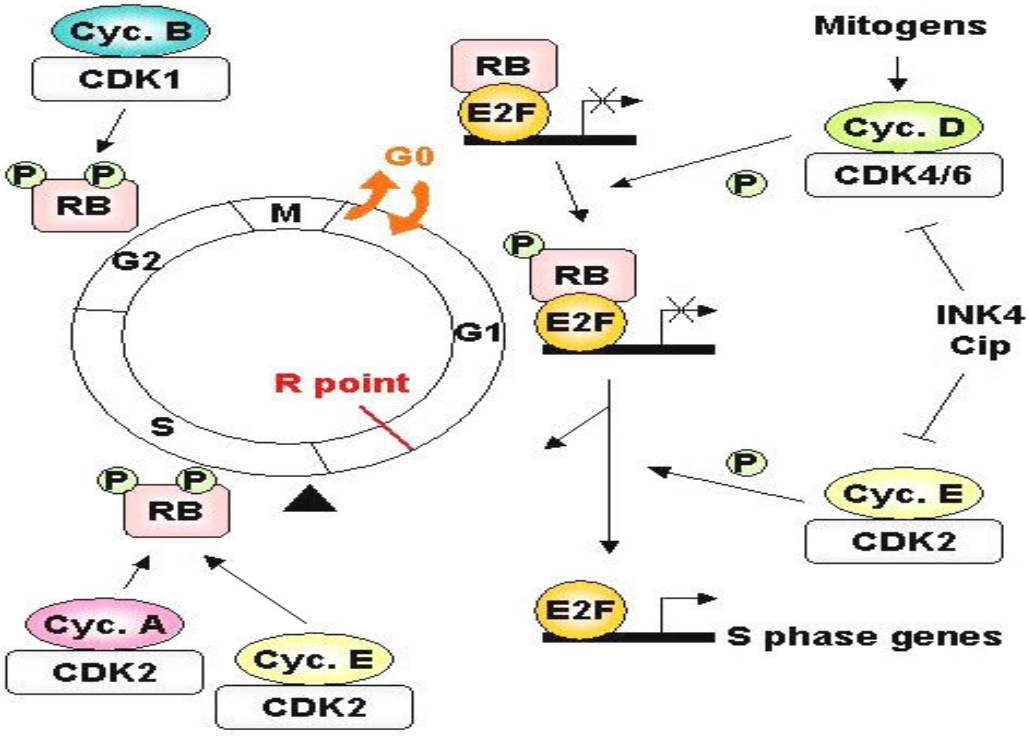
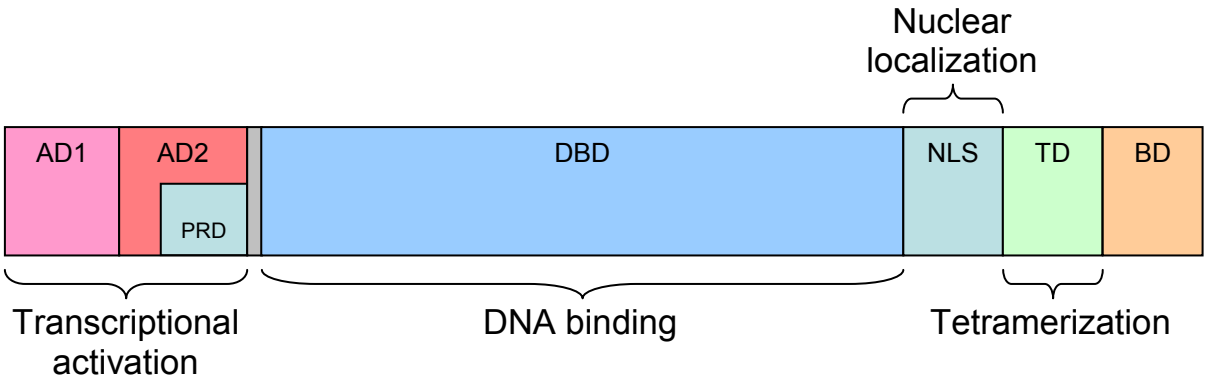


Figure 3: The Mammalian cell cycle (see text for explanation). P indicates phosphate groups. INK4/Cip indicates the INK4 and CIP family of Cdk-inihibitory proteins (CKIs).

1.2 p53 pathway

p53 is a tumoursupressor gene, and it is reported mutated in more then 50% of human cancers evidencing its role in tumourigenesis. The p53 protein was first described in 1979 when it was found to complex with SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). Its overexpression seemed to cause oncogentic transformation of cells. This misled the first investigators to regard p53 as a tumour antigen or an oncogene. Later, when wild-type p53 was found, the true nature of p53 was revealed. Wild-type p53 was often found inactivated in human tumour cells, and, when introduced into cells, it acted as a growth suppressor rather than an oncogene (Finlay et al., 1989). From the late 1980's, p53 was therefore considered a tumour suppressing gene, rather than a tumour promoting gene.



AD: Activation domain,
DBD: DNA binding domain
PRD: Proline rich domain,
NLS: Nuclear localization signal
TD: Tetramerisation domain
BD: Basic domain

Figure 4: The structure of the p53 protein.

P53 is a nuclear transcription factor of 53 k Dalton. It activates the transcription of several target genes involved in cell growth and apoptosis. The protein consists of 339 amino acid residues, devided into four structurally and functionally different domains. The acidic amino terminal consists of the first 42 amino acids, and is responsible for the transactivating properties of the protein. Without this domain, the induction of target genes cannot occur. Interaction with MDM-2 also occurs at this domain. Amino acids ranging from 102 to 292 form the central sequence-specific DNA-binding domain. This is the most common location of p53 mutations. Between these, a novel proline rich domain has been identified. The carboxy-terminal domain displays many functions, and can be further divided into the oligomerization domain and the basic C-terminus.

In broad terms, a wide variety of intrinsic and extrinsic stress signals engage the p53 network, resulting in marked accumulation and activation of the p53 protein. These signals all impact upon the cellular homeostatic mechanisms that monitor and control the fidelity of DNA replication, chromosome segregation and cell division. Among the stresses that activate p53 is damage to the DNA by γ – or UV irradiation, alkylation of bases, DNA cross-linking, depurination of DNA, reaction with oxidative free radicals, alteration of the deoxyribose sugar moiety and by other stress signals like spindle damage, hypoxia, nutrition deprivation, nitric oxide in inflammation, heat/cold shock and rNTP (Ribonucleoside triphosphate) depletion (for review see Levine et al. 2006). This large number of diverse cellular stress signals is fed into only a central, single node for monitoring, i.e. p53. The advantage of this arrangement is that one entity can act as an efficient integrator of information about all the different types of stress that can act upon the cell. On the other hand, giving a single gene and its product such a crucial role also makes the system vulnerable. Most cancers appear to induce loss of function of the p53 pathway, or as a result of mutations of genes that encode proteins that act upon p53 (Royds and Lacopetta, 2006).

The activation of p53 that occurs in response to stress signals can be defined as an increase in concentration of the p53 protein, and an increased activity of a p53 protein for transcription of a set of genes that have a p53 response element in their promotors. The levels of p53 are regulated mainly by its proteolytic turnover, and under normal conditions the protein has a short half-life of approximately 6-20 min. This is due to an E3 ubiquitin ligase known as MDM-2 (murine double minute 2). Under normal conditions MDM-2 monoubiquinates the p53 protein, exports it from the nucleus into the cytoplasm where it is ployubiquinated and degraded by the 20S proteasome. The exact mechanism of stabilization of p53 remains unclear, but it involves a series of post-translational modifications to both itself and MDM2 which facilitate dissociation of the p53-MDM2 complex (for review, see Lavin and Gueven, 2006). It is evident that multiple other proteins also influence p53 stability. Recently, two other ubiquitin ligases were shown to act upon the p53 protein, COP-1 (Coat protein complex 1) and PIRH-2 (Leng et al., 2003; Dornan et al., 2004). MDM2 is also capable of self ubiquination. Exposure of cells to stress reduces sumoylation of MDM2 and causes an increase in self-ubiquitination and degradation, thus favouring p53 stabilization. There is also evidence that post-translational modification of the MDM2 protein destabilizes its interaction with p53 and therefore contributes to p53 stabilization in response to stress. Studies have revealed that ATM phosphorylates MDM2 on Ser 395 in response to double strand DNA breaks, making it less capable of exporting p53 from the nucleus, suggesting the phosphorylation destabilizes p53-MDM2 interaction (Maya et al., 2001). ATM can also phosphorylate p53 directly on Ser15, and indirectly on Ser20 via Chk2 and thereby contribute to its stabilisation.

Another protein, MDMX, inhibits the transactivation activity of p53 (Marine et al., 2005). MDM2 and MDMX interact to protect p53 from MDM2 mediated degradation. This is achieved by prevention of nuclear transport by MDMX. On the other hand, MDMX stabilizes MDM2 by preventing its self-ubiquination. MDM2 and MDMX are functionally dependent in controlling p53. In absence of MDMX, MDM2 have a shorter half life and reduced capacity to control p53, while MDMX is dependent on its

binding to MDM2 to enter the nucleus to inhibit p53 function. In addition, it has recently shown that ATM-dependent phosphorylation of both MDM2 and MDMX lowers their affinity for the deubiquitinating enzyme herpes virus associated ubiquitin-specific protease (HAUSP), resulting in decreased activity and stability of these proteins, favouring p53 activation (Meulmeester et al., 2005).

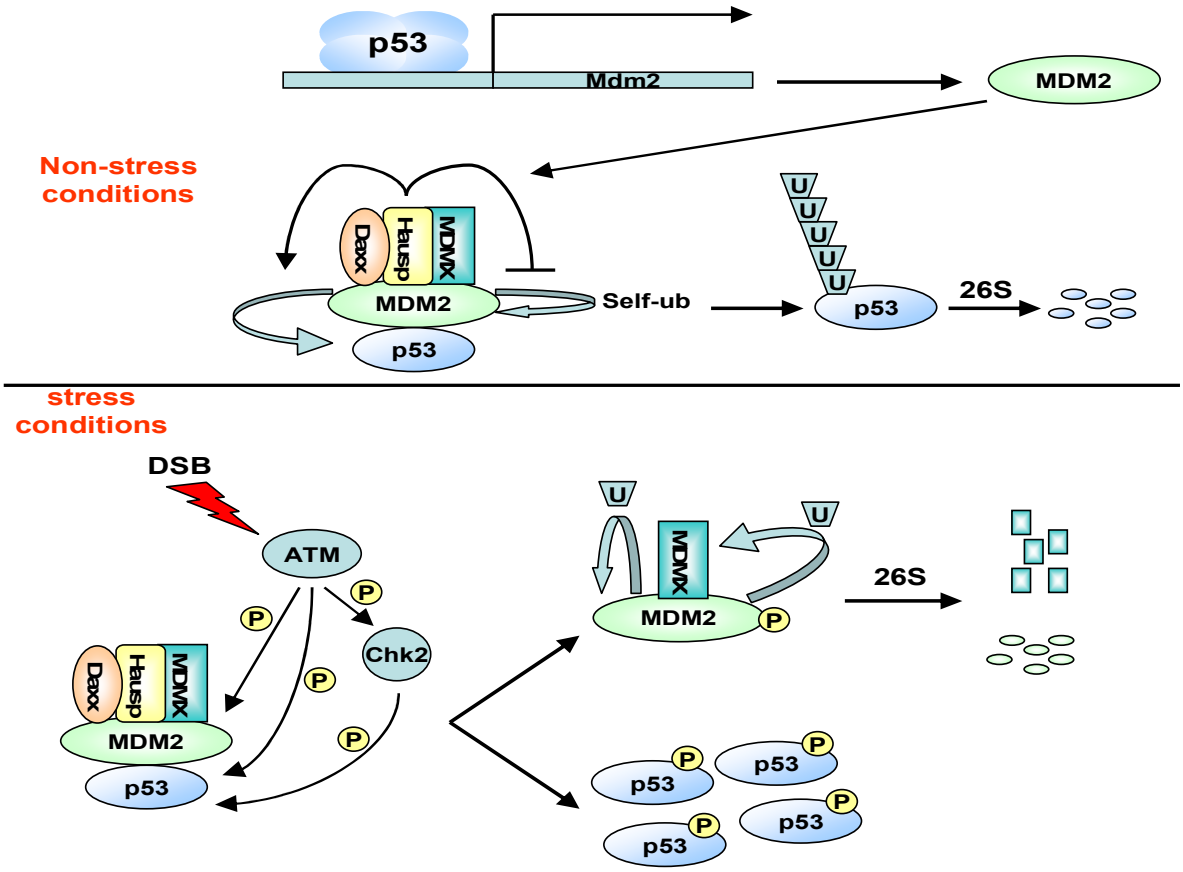


Figure 5: p53-mdm2 interaction under none-stress and stress conditions (see text for explanation).P indicates phosphate groups. 26S indicates proteolysis in the 26S proteasome. U indicates ubiquitin groups. DSB indicates DNA double strand breaks. Self-ub indicates self-ubiquination.

In addition to be regulated at the level of stability, the action of p53 can also be post-translationally mediated in response to stress signals. Thus, both the N-and C-terminal of p53 can be modified by phosphorylation, acetylation, methylation, ubiqitination and sumoylation. Clearly, the whole process of p53 activation is complicated and fine tuned to ensure that the protein is only activated with the appropriate response.

Several studies have suggested additional levels of the control of p53 protein, both at the transcriptional and the translational level, forming the basis of future research (for review, see Levine et al., 2006).

Once activated, p53 transcribes a number of genes that can be either stress- or tissue specific. MDM2 is one of them, causing alternating oscillating levels of p53 and MDM2 in the cell. There are three primary responses to a stress input signal by the p53 pathway; cell cycle arrest, apoptosis or cellular senescence. The way p53 brings about cellular senescence is not fully established, but one of the target genes of p53 that mediates G1 arrest is $p21^{(Cip1)}$. $p21^{(Cip1)}$ is phosphorylated and activated by p53 in response to cellular stress, and $p21^{(Cip1)}$ will in turn inhibit Cdk4/Cyclin D that normally phosphorylates pRB causing it do dissociate from E2F and thus inhibit the transcription of S-phase genes required for G1-S transition. $p21^{(Cip1)}$ also inhibits Cdk2/Cyclin E, to maintain the G1/S arrest. There is some controversy concerning whether p53 mediates G2 arrest. Another transcription product of p53 induced by DNA damage and some other stress signals is GADD45 (Growth arrest and DNA damage). GADD45 is a nuclear protein postulated to take part in both cell cycle arrest and DNA repair. Stabilized p53 also increases the expression of numerous pro-apoptotic genes (BAX, NOXA, PUMA etc) causing damaged cells to undergo apoptosis, and recently it has also been revealed that p53 has extranuclear apoptotic functions independent of transcription. Thus, p53 binds the anti-apoptotic BCL-2 proteins (Bcl-2 and Bcl-x) and activates the pro-apoptotic multi domain proteins (Bax and Bak) to induce mitochondrial outer membrane permeability (MOMP), cytochrome C release and subsequent apoptosis. Cells that have engaged p53-dependent apoptosis typically follow the intrinsic cell death pathway (see below), but it is also demonstrated that p53 can promote Fas-mediated (extrinsic) apoptosis by increasing the expression of Fas (Liu et al., 2006).

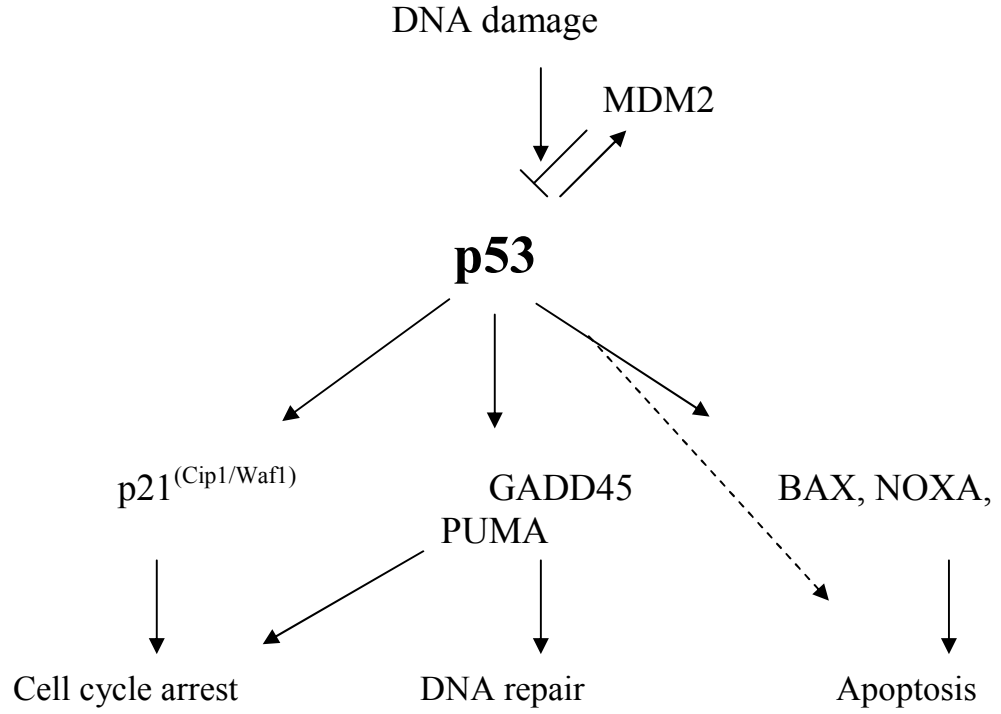


Figure 6: A simplified cartoon of p53 responses to DNA damage. Thin dashed arrow: transcription independent p53 mediated apoptosis.

1.3 Apoptosis

In general, cells die through one of two different pathways; necrosis or apoptosis. Necrosis is a passive and unregulated form of cell death caused either by stimuli that damage the cell membrane directly or by inhibition of the energy production in the cell (Endresen and Aarbakke, 1992). The necrotic cell swells, bursts and cell content leaks out causing inflammation in the surrounding tissue. Kerr and co-workers made the discovery of a form of cell death that was distinctly different from necrotic cell death occurring under pathological conditions (Kerr et al., 1972). In multicellular organisms, cells that are no longer needed or are a threat to the organism are destroyed by a tightly regulated cell suicide process known as programmed cell death or apoptosis. Apoptosis is a physiological from of cell death responsible for balancing the continuous renewal of the cells in the body and disturbances in the regulation of this process may be of importance in developing of diseases such as cancer, immune system disorders or neurodegenerative diseases.
The characteristics of apoptosis are cell shrinkage, chromosome condensation, fragmentation of DNA into 200bp fragments, and phagocytosis of the apoptotic cell. The process requires ATP, and no inflammation is induced in the surrounding tissues. The reason for this is that the membrane is never disrupted during apoptosis, and that apoptotic cells are recognized and phagocyted by macrophages.

There are two signalling pathways that trigger apoptosis (Chipuk and Green 2005). The intrinsic pathway is engaged by cellular stresses such as DNA damage, hypoxia or growth factor withdrawal. It directly impacts on the Bcl-2 family of proteins by transcriptional induction, repression or post-transcriptional modifications that act to repress or enhance function. The Bcl-proteins, Bax and Bak, then elicit mitochondrial outer membrane permabilization (MOMP), cytochrome c release and APAF-1 (apoptotic protease activating factor 1)-dependent pro-caspase-9 activation. Caspase-9-dependent cleavage then activates executioner caspases-3 and -7 responsible for the apoptotic hallmarks such as chromatin condensation, plasma membrane asymmetry and cellular blebbing. The Bcl-2 protein itself is also located in the outer mitochondrial membrane, and this protein forms complex with Bax or Bak to inhibit cytochrome C release and apoptosis.

The extrinsic pathway requires the ligation of death receptors by death ligands. For example, tumour necrosis factor (TNF) binds to its death receptor, TNFR1 which causes the recruitment of adaptor molecules, TRADD (TNFR1-assosiated death domain) and FADD (FAS-assosiated death domain). This causes pro-caspase-8 recruitment and activation. Executioner caspases-3 and -7 are then activated by caspase-8, resulting in cell death. Caspase-8 can also cleave and activate BID (BH3-interacting-domain death agonist), enabling it to co-engage the intrinsic pathway.

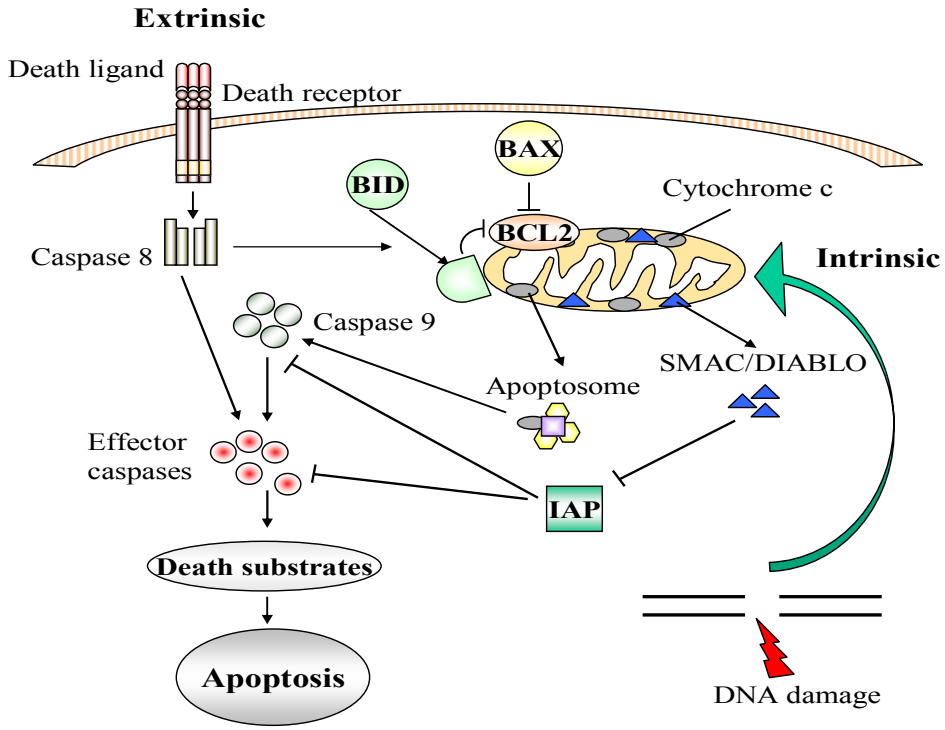


Figure 7: Apoptosis pathways (see text for explanation).

1.5 DNA damage caused by ionizing radiation

DNA damage is common in the life of a cell, and might lead to mutations, cancer, and cellular death. Damage to DNA induces several cellular responses that include: (a) removal of damage and restoration of the continuity of the DNA duplex; (b) activation of DNA damage checkpoints which arrests cell cycle and thereby allows the cell to repair the damage, and thus prevents the transmission of damaged or incompletely replicated chromosomes; (c) transcriptional response; and (d) apoptosis, which eliminates heavily damaged or deregulated cells. Defects in any of these processes may cause genomic instability (Sancar et. al, 2004). DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to DNA damage. These pathways are operative under normal growth conditions, and are amplified upon an increase in DNA damage. Whether there is a threshold for the amount of damage necessary to induce a checkpoint response is not known. The term checkpoint is defined, based upon the interstate transition that is being inhibited by DNA damage, as G1/S, intra-S and G2/M checkpoint.

If the DNA damage is double strand breaks caused by ionizing radiation, ATM (ataxia teleangiactasia mutated) is activated, functioning as a checkpoint sensor. Mutations in this gene can cause ataxia-teleangiectasia (A-T), a condition characterized by cerebellar degeneration, immunodeficiency, genome instability, clinical radiosensitivity and cancer predisposition, indicating a central role in regulation of cell proliferation. When activated, ATM phosphorylates many proteins, including Chk2 (Thr68), p53 (Ser20) and itself. These phosphorylations result in two signal transduction pathways, one to initiate and one to maintain G1/S-phase arrest. The initiating process is phosphorylating of Chk2, which in turn phosphorylates Cdc25 phosphatase, causing its deactivation by nuclear exclusion and ubiquitin-dependent proteolytic degradation. This results in the accumulation of the phosphorylated, inactive form of Cdk incapable of phosphorylating Cdc45 to initiate replication. This rapid response is followed by the p53 mediated maintenance of G1/S arrest.

ATM directly phosphorylates Ser15 of p53 and Ser20 indirectly via Chk2. This phosphorylation inhibits the nuclear export and degradation of p53, resulting in increased levels of p53. p53 then activates its target genes, including the Cdk inhibitor $p21^{(Cip1)}$, which inhibits transcription of S-phase genes as described for "The RB pathway". This results in maintenance of the G1/S arrest. In addition to cell cycle arrest, ionizing radiation damaging cells beyond repair may also lead to their elimination by p53-mediated apoptosis (see above). Loss of either of these functions could contribute to tumorigenesis. Cells that fail to arrest in G1 after DNA damage are more likely to propagate mutations, and in the absence of p53, damaged cells may not be eliminated from the tissue by apoptosis (Enoch, T. & Norbury, C., 1995).

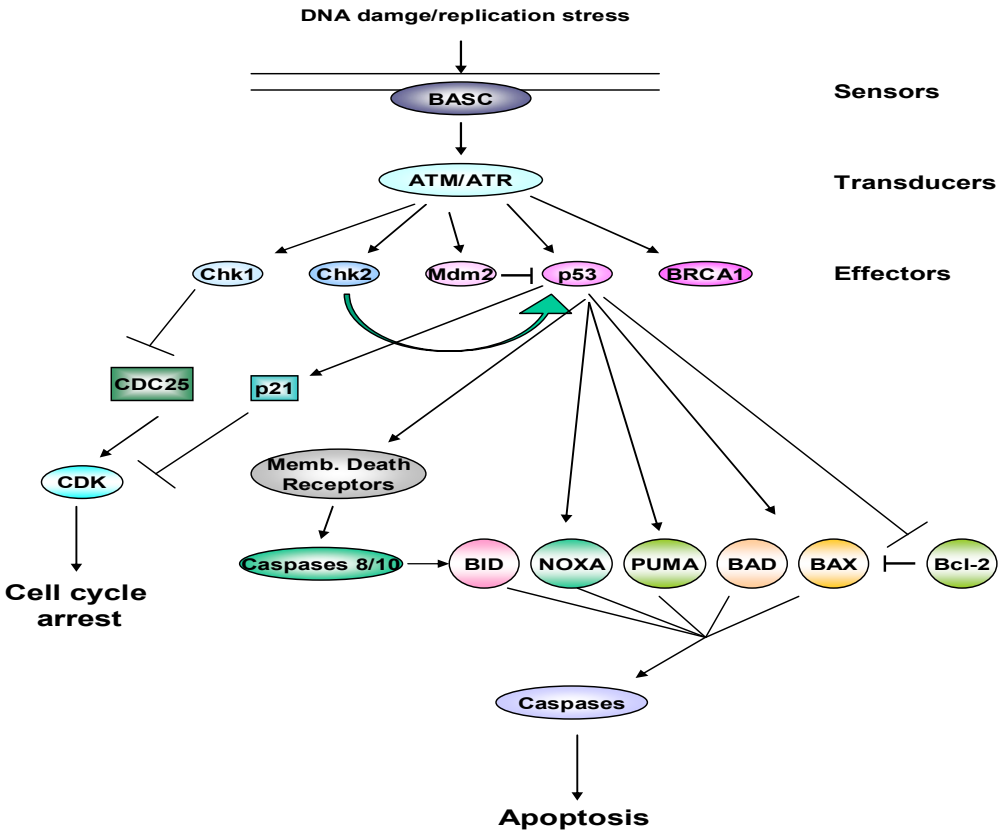


Figure 8: DNA-damage response.

1.5 cAMP/PKA pathway

The second messenger concept of signalling was born with the discovery of cyclic adenosine monophosphate (cAMP) and its ability to influence metabolism, cell shape and gene transcription via reversible protein phosphorylations. cAMP was first identified as a small intracellular mediator in the 1950s. Since then, it has been found to act in this role in all prokaryotic and animal cells studied. cAMP is synthesized from adenosine-triphosphate (ATP) by a plasma-membrane-bound enzyme named adenylyl cyclase (AC) that removes two phosphate groups as pyrophosphate. cAMP is unstable in the cell because it is rapidly and continuously destroyed by one or more cyclic AMP phosphodiesterases (PDA) that hydrolyze cAMP to adenosine 5'-monophosphate (5'-AMP). Extracellular signals such as hormones, growth factors and neurotransmitters can therefore by inducing rapid synthesis and removal of cAMP cause levels in the cell to change by more than twenty fold in seconds.

All receptors that act via cAMP are coupled to a stimulatory G protein, which activate AC and thereby increase cAMP concentration. It was long thought that the only target of cAMP in the cell was cAMP-dependent protein kinase (PKA). In recent years it has become clear that even though it mainly exerts its effects by this pathway, not all effects of cAMP in animal cells are mediated by general activation of PKA. At present, cAMP is known also to directly regulate ion channels and the ubiquitous Rap guanine exchange factors Epac1 and 2 (extracellular signal-regulated kinase)

(Kopperud et al. 2003). In inactive state, PKA in cytosol consists of a complex of two catalytic subunits and a regulatory subunit dimer to which cAMP can bind to (Corbin et al. 1978). Binding of cAMP to the regulatory subunits result in a conformational change, causing these subunits to dissociate from the catalytic subunits, and thus activating their catalytic kinase activity. The released catalytic subunits then move into the nucleus, where they phosphorylate gene regulatory proteins, while the regulatory subunit remains in the cytoplasm. The substrates for PKA differ in different cell types, which explain why the effects of cAMP are dependent on the cell type.

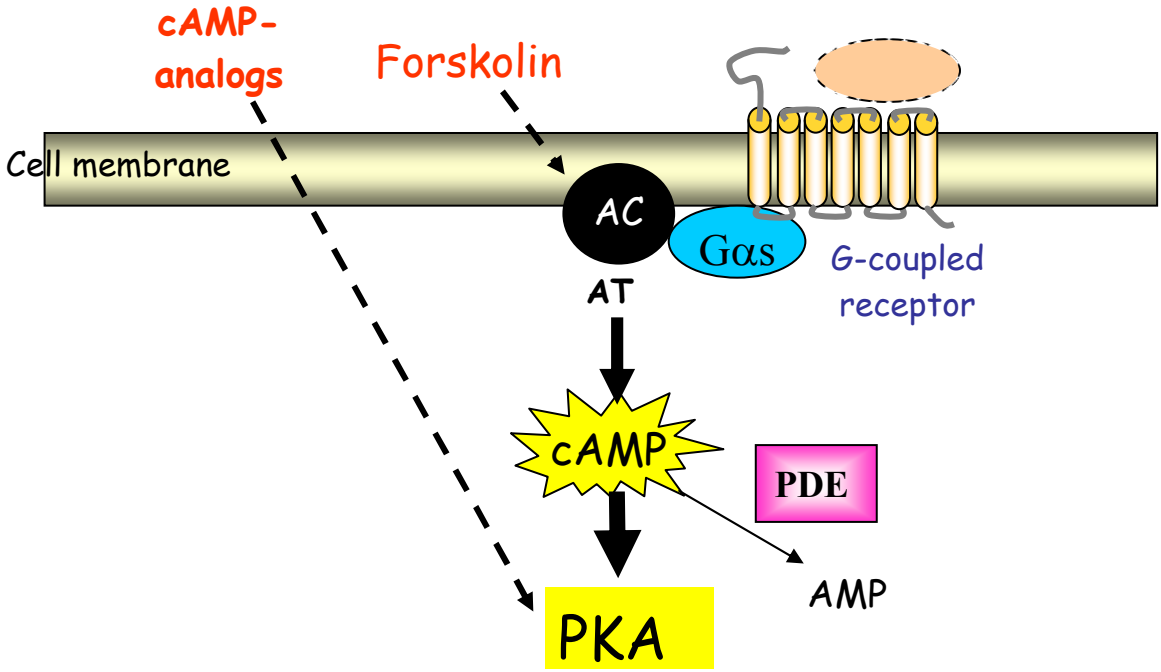


Figure 9: cAMP-PKA pathway. In order to activate PKA in cells we have used the two components forskolin and 8-CPT-cAMP.Forskolin is a deterpine compound isolated form plants, and it induces increased intracellular levels of cAMP by directly activating AC (Seamon & Daly 1981). 8-CPT-cAMP is a membrane permeable analogue of cAMP.

1.5.1 cAMP and growth control

cAMP exerts an antiproliferative effect on a number of cell types, including lymphocytes (Blomhoff et al. 1987), proposed to be mediated by its ability to inhibit G1/S transition. Evidence of a new mechanism whereby cAMP might inhibit cellular proliferation was recently provided by our group. It was shown that elevation of intracellular levels of cAMP inhibits DNA replication and arrests the cells in S-phase. This inhibition is associated with increased binding of $p21^{(Cip1)}$ to Cdk2-cyclin complexes, inhibition of Cdk kinase activity, dephosphorylation of RB and dissociation of PCNA (proliferating cell nuclear antigen) from chromatin in S phase cells (Naderi et al. 2005).

1.6 The immune system

The main purpose of the immune system is to protect the organism from infections. The immune response is divided in two; the innate (non-specific) and adaptive (specific) immune response. The innate immunity is the first line of defence against foreign agents. It is mediated by cellular elements, such as monocytes and macrophages, and components of the complement system. These components do not require previous exposure to foreign agents. The adaptive immunity is mediated via lymphocytes, and is characterized by specificity for antigen and immunological memory. T-lymphocytes are responsible for the cellular part of the specific immune response, whereas B-lymphocytes mediate humoral immunity through production of antibodies. The innate response is rapid, but can sometimes damage normal tissue due to lack of specificity. The adaptive immune response take several days or weeks to develop, but is precise and due to its immunological memory, subsequent responses are more vigorous and rapid. There are interaction between B-cells and T-cells both during development and activation of cells and during the adaptive immune response.

1.6.1 B-cell development and response

B-cell development takes place in the bone marrow. Here, gene segments encoding the variable (V) region of antibody molecules are assembled by somatic recombination. As a large number of gene segments (V,D,J) are available, each B-cell has a unique B-cell receptor (BCR) which represents a clonal marker. B-cells expressing a functional BCR are released into the periphery as mature, antigen-naive B-cells. If antigen is recognized by the naive B-cell, it will bind, and if T-cell help is available, initiate activation of the B-cells in the T-cell zone of lymphoid organs. Activated B-cells then migrate into B-cell follicles, start to proliferate and differentiate into centroblasts, and thereby establish germinal-centres.

In the proliferating centroblasts the process of somatic hypermutation (SMH) in the V region genes is established, and thus generates new antibody variants. Centroblasts then differentiate into resting centrocytes, and only cells with acquired affinity-increasing mutations of their BCR are able to interact with the germinal-centre T-cells and follicular dendritic cells (FDCs) and thereby receive survival-signals. The remaining undergo apoptosis. The selected germinal-centre B-cells then undergo repeated rounds of replication, mutation, and selection, and the immunoglobulin genes of many of the cells are also remodelled by class switching.
Finally the selected germinal-centre B-cells differentiate into either memory B-cells or plasma cells and leaves the germinal centre.

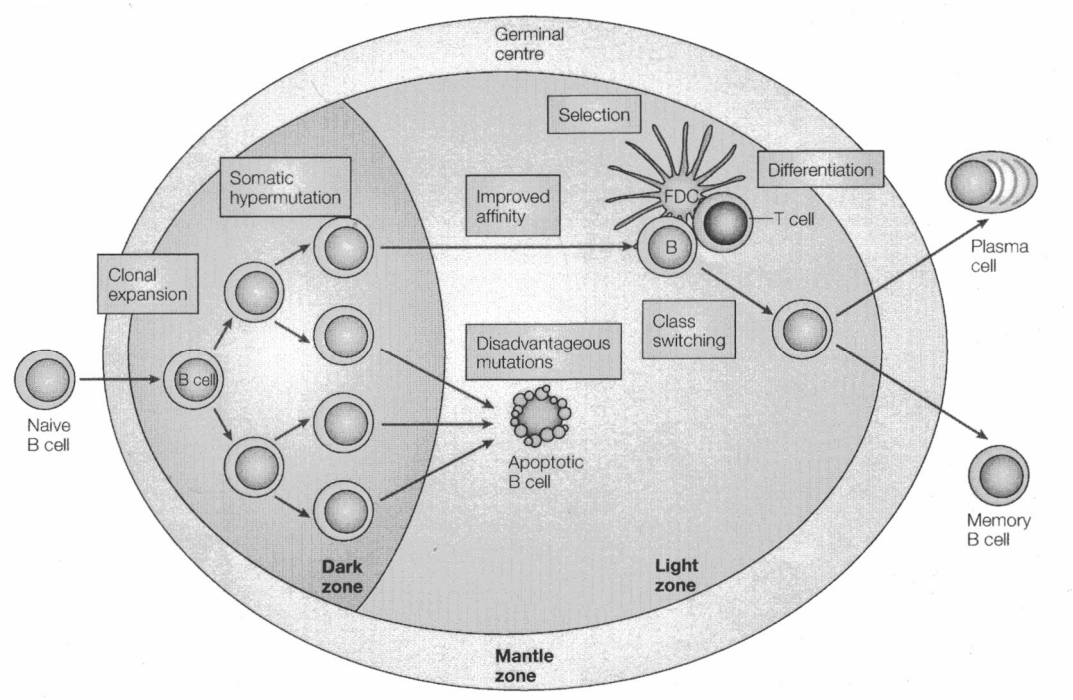


Figure 10: B-cell developmental pathways. FDC indicates follicular dendritic cell. B indicates B-cell. (Küppers, 2003)

1.6.2 The B-lymphoid cell line Reh

A population of cells that is able to propagate indefinitely in culture is called a cell line. As normal B-lymphocytes have very limited lifespan in culture, cell lines derived from normal and malignant lymphocytes have become a valuable tool in research. In the experiments in this paper, the lymphoid cell line Reh was used as a control against EBV-transformed B-lymphocytes. This B-lymphoid line was established in 1974 from the blood of a 15-year old North African girl with acute lymphoblastic leukaemia (Rosenfeld et al, 1977). Like normal B-lymphocytes, the Reh cells express the CD19 marker, and the cells are EBV-negative.

1.7 Epstein- Barr virus

Epstein- Barr virus (EBV) was the first candidate human tumour virus. It is a double stranded DNA virus genome of 172 kb, belonging to the γ herpes virus family. It mainly targets B-lymphocytes, but in some circumstances EBV also infects other cells, like T-cells or epithelial cells. The virus was first identified in 1964 from examining electron micrographs of cells cultured from Burkitt's lymphoma (Epstein et al.1964). EBV is the most successful human pathogen because it latently infects greater then 90% of the human population. Hosts are usually affected in early childhood as an asymptomatic infection. Like all herpes viruses, EBV is able to persist in the host for life, but in the majority of healthy carriers the virus causes no disease. This is because a delicate balance between the host immune system and the virus is maintained. Disruption of this balance may lead to the development of EBV-associated disease. It therefore seems that EBV has the potential to be highly pathogenic yet rarely manifests this potential. Studies over the last several years show

that this is because EBV has the unique ability to transform resting B-cells into permanent, latently infected lymphoblastoid cell lines (LCLs), and therefore persists in resting, non proliferating memory cells (Babcock et al., 1998).

1.7.1 EBV latent genes and transformation

In EBV-transformed LCLs, every cell carries multiple extrachromosomal copies of the viral episome and constitutively expresses a limited set of viral gene products. EBV transits the epithelium and infects naive B-cells in the underlying tissue, where it expresses a set of latent genes that causes the cell to become activated and proliferate as though it was responding to antigen. This growth program/latency III consists of six EBV nuclear antigens (EBNAs 1,2,3A,3B and LP) and three latent membrane proteins (LMPs 1, 2A and 2B). These proteins have all the necessary activities to push the B-cell to become an activated blast without any external signalling (for review, see Young and Rickinson, 2004).

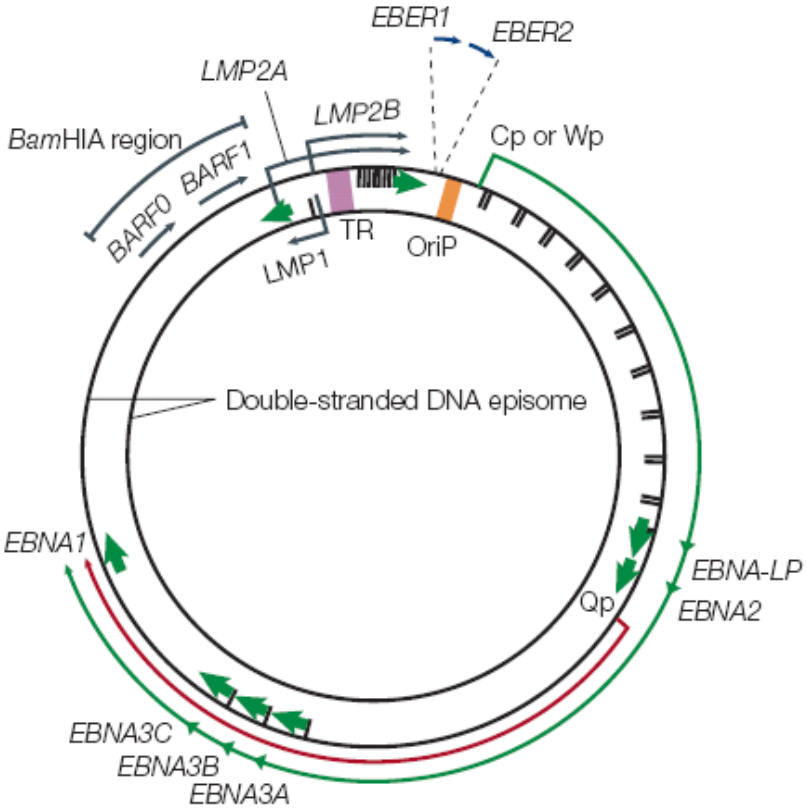


Figure 11: The Epstein-Barr virus genome. Diagram showing the location of the EBV latent genes on the double stranded viral DNA episome. OriP indicates the origin of plasmid replication. TR indicates the terminal repeat region. EBNA indicates the latent EBV nuclear antigens. LMP indicates the latent membrane proteins. The blue arrow represents the highly transcribed non-polyadenylated RNAs EBER1 and 2. The long outer green arrow represents EBV transcription during latency III, always starting at the Cp or Wp promoter. The inner red arrow represents the EBNA1 transcript, which originates from the Qp promoter during latency I and II. Transcripts from the BamHIA region can be detected during latent infection, but no protein arising form this region has been definitively identified. The locations of the BARF 0 and 1 are also shown (Young and Rickinson, 2004).

Once activated, the cell migrates to the follicle where the viral transcription program changes, so that only three latent proteins are expressed: EBNA1 (required to replicate viral DNA), LMP1 and LMP2 (the default program/latency II). LMP2 alone will push B-cells to form a germinal centre in the mucosal follicle. LMP1 and LMP 2 can drive immunological gene mutation and isotype switching, respectively, and LMP1 downregulates the expression of bcl-6 (germinal centre regulatory transcription factor), the signal for a memory cell to exit the germinal centre. This implies a coordinated process in which LMP2 is turned on before LMP1 during the germinal centre reaction. Once in the periphery, the LCLs shut down all viral protein expression (EBNA1 only program/latency I), and appear to be maintained as normal memory B-cells (for a review, see Thorley-Lawson, 2006).

Infectious mononucleosis (IM) is generally the result of delayed primary infection with EBV characterized by the triad of fever, lymphadenopathy and pharyngitis (Papesch & Watkins, 2001). These symptoms typically occur in young adults, but are occasionally seen in children and older adults. IM is rare in nonindustrial countries, where infection almost invariably occurs asymptomatic in early childhood. A classical feature of IM is blood lymphocytosis, consisting mainly of CD8+ T-cells, and this immune response is thought to cause the clinical symptoms. In the early stages of the acute infection, the number of LCLs in the blood reaches staggering proportions, with 50% or more of memory cells being infected. Classic acute IM resolves in 2 to 6 weeks, but relapses can occur in the first 6- 12 months, and IM may be linked with a prolonged fatigue syndrome and depression. After this time, the level of infected cells appears to be relatively stable over many years (for review see Williams & Crawford, 2006). There are no longer viral proteins expressed that can cause the cell to divide, and because EBNA1 represents the only point of immune attack of the memory cells, it has evolved to be poorly recognized by the immune system (Levitskalya et al., 1995).

The EBV might promote the development of gastric cancer (Osato & Imai, 1996), nasopharyngeal carcinoma (Henle & Henle, 1976), and B-cell malignancies, including Burkitt's lymphoma (Epstein et al., 1964), Hodgkin's lymphoma (Deacon et al, 1993), post-transplant lymphoproliferative disease (Swinnen, L.J., 1999), and AIDS-associated lymphomas. The origin of these tumours can be understood as arising from specific stages of the EBV life cycle, and appear to be associated with disturbances in the immune system. The first three types of B-cell malignancies have been studied most extensively, and will be discussed below.

1.7.2 Burkitt's lymphoma

EBV was discovered in cultured tumour cells from patients with the endemic form of Burkitt's lymphoma. The lymphoma was first described in children in equatorial Africa and New Guinea where malaria is holoendemic (Burkitt, 1962). EBV is present in all cases of 'endemic' lymphomas, in up to 85% of cases in areas with intermediate incidence, but only in 15% of the low-incidence sporadic tumours seen in children in the developed world. Burkitt's lymphoma is also common among adults with HIV, often arising as the first AIDS-defining illness in relatively immunocompetent patients. A hallmark of Burkitt's lymphomas is the translocations of the proto-oncogene MYC into one of the immunoglobulin loci, resulting in constitutive activity of this transcription factor. In addition, many tumours have mutations in the p53 gene

or other defects in the p53-ARF pathway, as well as mutations in the putative tumour suppressor gene retinoblastoma-like 2(RB2). Burkitt lymphoma cells phenotypically resemble centroblasts. Many lymphoma-cells also show evidence of active somatic hypermutation – and thereby support the suggestion that they originate in germinal centres (Klein et al., 1995). Besides the EBERs and BARTs, only EBNA1 protein is expressed (latency I/EBNA1 only program). Its oncogenic potential is debated. Several findings indicate a possible role for the EBERs, but how EBV contributes to the pathogenesis of Burkitt's lymphoma still remains a matter of speculation.

1.7.3 Hodgkin's lymphoma

Acute EBV-infection in adolescent-adult can give rise to IM, long known to be a risk factor for Hodgkin's lymphoma (HD). HD is characterized by atypical, large tumour cells known as Reed-Sternberg cells (HRS) (Küppers, 2002). These usually represent less than 1% of the tumour tissue, the rest is non-malignant T-cells, B-cells, eosinophils and others. There is a classical form of Hodgkin's Lymphoma, accounting for 95% of cases, and a lymphocyte predominant form. The HRS of the latter is always EBV negative, whereas in 40% of the classical form in the western world EBV is detected. This can approach 80% in developing countries and up to 100% in AIDS-related HD (for review, see Andersson, 2006)

In EBV-positive cases, three EBV proteins are expressed; EBNA1, LMP1 and LMP2A (latency II/default transcription programme). Single-cell molecular analysis showed that HRS cells, in nearly all cases derive from B-cells; less than 5% have T-cell origin. The rearranged Ig V genes of HRS are somatically mutated, but lack intraclonal diversity, indicating that the SHM machinery is silenced in the tumour cells. In ~25% of cases, there are non-sense mutations or deleterious deletions in originally functional V gene arrangements. Normally, such 'crippling' mutations would sentence the B-cell to apoptotic destruction in the germinal centre, and therefore HRS cells presumably derive from pre-apoptotic germinal centre B-cells that were rescued from apoptosis by some transforming event (s). As a lot of other mutations can target a cell for apoptosis, it is postulated that the HRS cells lacking recognizable crippling mutations also derive from the pool of pre-apoptotic germinal-centre B-cells with disadvantageous mutations. (Küppers, 2003).

1.7.4 Post-transplant lymphoproliferative disease

T-cell immunosuppressive therapy given to patients after transplantation renders patients susceptible to post-transplant lymphoproliferative diseases (PTLDs). Most PTDLs arise as polyclonal or monoclonal lesions within the first year after the procedure. These PTLDs are almost always of B-cell origin and EBV-positive, most likely because suppression of T-cell response allows uncontrolled proliferation of EBV-transformed B-cells. The majority expresses the latency III programme/Growth programme, but more restricted gene-expression patterns are also observed. Transplant cohorts continue to show a significant, albeit lower risk of proliferative disease well beyond the first year, but this represents a more heterogeneous group, and the number of EBV-associated cases can fall below 50%. Ig-gene sequencing show that PTLDs can arise from various stages of the B-cell differentiation pathway (for review, see Lim et al., 2006).

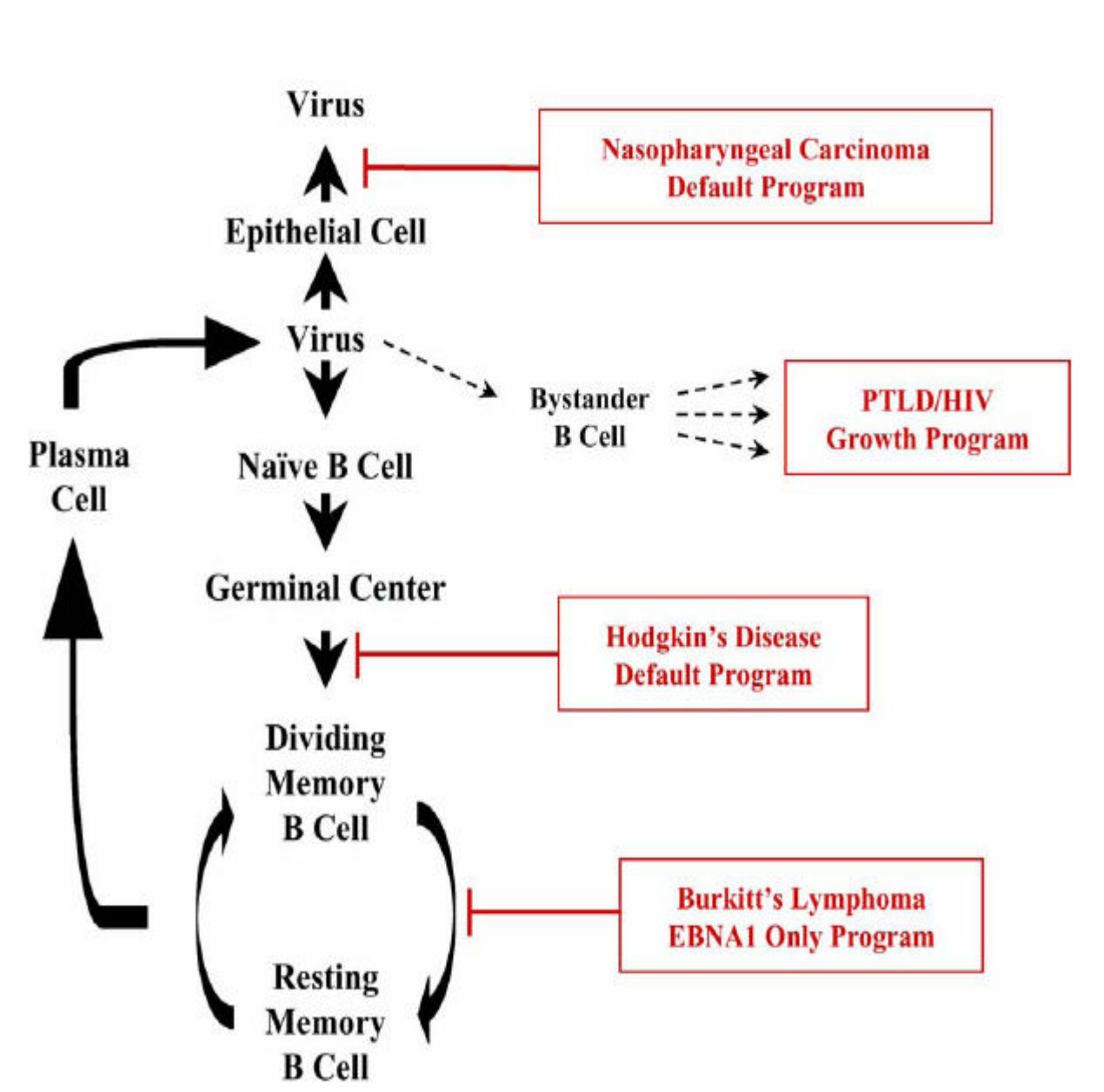


Figure 12: The putative checkpoints in the EBV life cycle that gives rise to tumours. The normal events in healthy carriers denoted in black. EBV infects naive B-cells in the Waldeyer's ring, and these cells can differentiate into memory cells and out of the cell cycle, and are therefore not pathogenic. **PTLD** indicates post-transplant lymphoproliferative disease. If another cell than the naive B-cell in the Waldeyer ring becomes infected, it will express the latency III/growth program and continue to proliferate because it cannot differentiate out of the cell cycle (thin dashed lines). Normally these bystander B-cell blasts would be destroyed by CD8+ T-cells, but if the immune response is suppressed, they then can grow onto PTLD. A bystander B-cell could also arise if a latently infected germinal centre or memory cell fortuitously switched on the latency III/growth program. **Hodgkin's disease**: arises from EBV-infected cells that are blocked at the germinal centre stage resulting constitutive expression of the default program/latency II. **Burkitt's lymphoma**: evolves from germinal centre cells that are entering the memory cell compartment, but are stuck proliferating and consequently express the latency I/ EBNA1 only program (Thorley-Lawson, 2006).

2. AIMS OF THIS THESIS

Previous work in our lab has shown that elevation of intracellular cAMP leads to G1 arrest in normal and malignant lymphoid cells (Blomhoff et al., 1987), and recently it was also shown that elevation of intracellular levels of cAMP inhibits DNA replication and arrests the cells in S-phase (Naderi et al. 2005).

Many anticancer agents that act via DNA damage-induced apoptosis (cytotoxic dugs and γ-irradiation) depend on DNA replication to exert their function and it could therefore be anticipated that elevation of cAMP would inhibit such DNA damage-induced apoptosis. p53 is a tumour suppressor and an important downstream mediator of DNA damage responses. Upon DNA damage, p53 is stabilized and activated to induce transcription of genes involved in growth arrest, DNA repair and apoptosis. Our lab has recently demonstrated that elevation of intracellular cAMP indeed leads to inhibition of DNA damage induced apoptosis (Naderi et al., 2005), and our results so far have shown that cAMP inhibits DNA damage-induced apoptosis by downregulation of p53 at the level of protein stabilisation (Hallan Naderi et al., manuscript in preparation).

Epstein-Barr virus is a DNA tumour virus belonging to the γ-herpes virus family. It can cause different types of cancer and proliferative disorders including Burkitt's lymphoma (Epstein et al., 1964), nasopharyngeal carcinoma (Henle & Henle, 1976), gastric cancer (Osato & Imai, 1996), Hodgkin's lymphoma (Deacon et al, 1993), and post-transplant lymphoproliferative diseases (Swinnen, 1999). Several years ago we showed in our lab that EBV-transformed B-cells no longer were inhibited by TGFβ (Blomhoff et al., 1987). The mechanism for this has later been described (For review, see Mori et al., 2003). Recently, we have shown that EBV- infection of normal B-lymphocytes also abolishes the antiproliferative effect of cAMP in these cells, and it seems as though cAMP in EBV-transformed B-cells no longer can inhibit DNA replication (Kloster et al., manuscript in preparation).

Since cAMP-mediated inhibition of DNA damage-induced apoptosis seems to be linked to inhibition of DNA replication and cAMP-mediated inhibition of DNA replication is reduced in EBV-transformed B-cells, we now have the following aims;

1) To unravel whether EBV-infection of B-cells prevents cAMP-mediated inhibition of DNA damage-induced apoptosis triggered by γ-irradiation.

2) To establish the mechanisms involved in EBV-mediated regulation of DNA damage- induced apoptosis.

3. MATERIALS AND METHODS

3.1 Materials

Chemical	Producer
Acidic acid	Merck
Acrylamide/Bis	Sigma
Ammonium persulphate	Shelton scientific
Aprotinin	Sigma
β-mercaptoethanol	Sigma
β-glycerophosphate	Sigma
Bio-Rad Protein assay	Bio-Rad
Bromophenol blue	Bio-Rad
Di-sodium hydrogen phosphate (NaH_2PO_4)	Prolabo
Fetal Bovine Serum	Gibco/BRL
Forskolin	Calbiochem
γ-globulin	Sigma
Glycerol	Prolabo
Glycine	Sigma
HCl	Merck
Isopropanol	Arcus
Leupeptin	Sigma
Methanol	BDH
Molecular weight standard	Bio-Rad
Non fat dried milk	Nestle
Penicillin Streptomycin	Gibco/BRL
PMSF	Sigma
Ponceu S	Sigma
Propidium Iodide	Sigma
RPMI 1640	Gibco/BRL
Sodium chloride (NaCl)	BDH laboratories
Sodium deoxycholate	Sigma
Sodium dodecyl sulphate (SDS)	Sigma
Sodium Fluoride (NaF)	Merck
Sodium orthovandate (Na_3VO_4)	Sigma
TEMED	Bio-Rad
Tris base	Angus Buffers & Biochemicals
Triton X-100	Sigma
Tween20	Sigma
8CPT-cAMP	BIOLOG

Antibodies

P53 total	Cell signalling
P53 S15	Cell signalling
P53 S20	Cell signalling
Actin	Santa cruz
Chk2 T68	Cell signalling
Chk1 S317, S345	Cell signalling
ATM	Rockland
Goat-Anti-Mouse IgG	Bio-Rad
Goat-Anti-Rabbit IgG	Bio-Rad

Kits

ECL Western blotting analysis system	Amersham Pharmacia Biotech
Bio-Rab protein assay	Bio-Rad

Equipment

Beckman J2-21 centrifuge	Beckman
Biofuge fresco table top centrifuge	Haraeus
Cell culture flasks	NUNC
Cell culturing plates (6-12-24 well)	BC;Falcon
Centrifuge tube (15 and 50 ml)	Merck
CO_2-incubator	Nuaire™
Coulter® Microdiff 18 (Cell counter)	Dan Meszantsky
Eppendorf tubes (micro tubes 1,5 ml)	Axygen Scientific
Erlenmeyer bottles	Pyrex
FaCSCaliber	Becton Dickinson
Falcon tubes (5ml)	Becton Dickinson
Gamma-cell®3000 Elan	MDS Nordion
Glacpack™	Andvord
Heating block	Dan Meszantsky
Hybond™ ECL™ Nitrocellulose membrane	Amersham Pharmacia Biotech
Hyperfilm™ MP	Amersham Pharmacia Biotech
Kodak X-Omatic cassette	Kodak
Light microscope with 40x objective	Nikon
ParafilmM	Kebolab
Platform shaker	Edmund Blücker
Rock-n-roller	Labinco
Polystyrene Round-Bottom tube (5ml)	Falcon
Rotator mixer	Stuart scientific
Semicro disposable cuvettes (1,5ml)	Pharmacia Biotech
Sigma 4K15 centrifuge	Sigma
Spectrophotometer, Ultrospec 3000	Pharmacia Biotech
Trans-Blot® SD Semi-Dry Transfer Cell	Bio Rad
Water bath	Julabo
Whatman® Chromatography paper 3 MM	Whatman

3.2 Isolation and culturing of cells

When isolating cells and keeping them in culture, it is important that all equipment used and all reagents in contact with the cells are sterile. This is accomplished by using an antiseptic technique at all times. In addition, antibiotics are added to prevent bacterial growth. The cells are grown in an incubator at 37 ˚C with 5% CO_2 and humidified air to stimulate an *in vivo* situation.

3.2.1 Isolation and EBV-transformation of B-lymphocytes

Normal human lymphocytes are isolated from buffy-coats derived from volunteer blood donors at Ullevål University hospital. The erythrocytes and plasma are removed for clinical use, and what is left of the blood is called a buffy-coat. When studying B-lymphocytes it is important to remove contaminating cells that can affect the functions of B-lymphocytes by cell-cell-contact or secretion of cytokines. B-cells are isolated from the buffy-coat by positive selection .This is done by using magnetic beads coated with antibodies against the CD19 antigen that is expressed on the surface of almost all B-cells, with the exception of plasma cells.

EBV-transformation of B-lymphocytes is performed by resuspension of the isolated lymphocytes in RPMI medium and adding EBV supernatant from B 95/8 monkey cells and cyclosporine A. The medium is changed every third day for the first 14 days by removing 1 ml of old medium and replacing it with fresh medium containing cyclosporine A. After another week, the cells can be grown in culture flasks as other cells in suspension, and can be used in experiments.

Since I did not isolate the B-cells of infect the cells with EBV myself, I will not explain these methods in detail.

3.2.2. Culturing lymphoid cell lines and EBV-transformed B-cells.

The lymphoid cell line Reh and the EBV-transformed B-cells are grown in complete RPMI medium. This medium contains inorganic salts, amino acids, vitamins, glucose, glutathione and pH-indicator. In addition there are growth factors that all cells need to grow and proliferate. The cells are cultured in 10% FBS.

Equipment and solutions
Complete RPMI medium
Cell culture flask
CO_2 incubator

<u>RPMI medium with 10% FBS</u>

RPMI1640	500 ml
Heat inactivated FBS	50 ml
Penicillin 5000 IE/ml- Streptomycin 5 mg/ml	10 ml

The medium is stored at 4 ˚C

Procedure:
EBV-transformed human B-lymphocytes have a doubling time of approximately 36 hours. The cell cultures are diluted so that the cell concentration is kept between 0,5 – 1,0 x 10^6 cells/ml. Reh cells have a doubling time of about 24 hours, and the optimum density of the cells is 0,15 – 1,5 x 10^6 cells/ml. When used in experiments, Reh cells are split to 0,4 x 10^6 cells/ml the day before use. Otherwise the cultures are split every 2 or 3 days with RPMI medium.

3.2.5 Cell counting

Cells are counted in an automatic cell counter, Coulter® Microdiff 18 ,which counts cells by detecting and measuring changes in electrical resistance when a particle in a conductive liquid passes through a small aperture. As each cell passes, it impedes the current and causes a measurable pulse. The number of pulses signals the number of cells.

3.3. Cell irradiation

To induce DNA damage, the cells were exposed to γ-irradiation.

Equipment and solutions	
Gamma-cell®3000 Elan	RPMI/PS/10 % FBS
15 ml tubes	Cell culture flask
12-well cell culture plate	CO_2 incubator

Procedure:
The EBV- and Reh-cells were divided into smaller culture flasks (5- 10 ml), and exposed to ionizing radiation in the Gamma-cell®3000 Elan-apparatus. Reh-cells were treated with 10 Gy, and EBV-cells with 40 Gy. Following irradiation, the cells where incubated for another 4 hours at 37˚C . 0, 5 – 1 ml of each sample were then transferred to a 12 well culture plate, and incubated for 20 more hours before used in the Scatter/PI-analysis. The rest of the samples were used in the Western blot analysis after the initial 4 hours.

3.4 Measuring cell death

3.4.1 Scatter analysis

This analysis is used to measure the distribution of viable and dead cells. A fluorescence activated cell sorter (FACS) measures and analyses optical properties of single cells passing through a focused laser beam. When cells pass through the laser beam, they disrupt and scatter the laser light, which is detected as forward scatter and side scatter. Forward scatter light is related to cell size while side scattered light is an indicator of the cell's granularity. Apoptotic cells are smaller than viable cells, and more granular due to shrinkage of the cells, chromatin condensation, fragmentation of nuclei and the formation of apoptotic bodies. Necrotic cells will be larger than viable cells due to swelling. Thus, by this method it is possible to separate cells into viable cells, apoptotic cells and necrotic cells.

3.4.2 PI-staining of cells

By staining cells with Propidium iodide (PI) it is possible to distinguish between dead and viable cells. PI is a DNA-binding dye taken up by necrotic and late apoptotic cells. Viable and early apoptotic cells exclude PI because they have an intact nuclei membrane. PI-staining of the cells is detected as red fluorescence in a flow cytometer equipped with an argon laser.

Equipment and solutions	
24/12 well cell culture plate	RPMI/PS/10% FBS
Polystyrene round bottom tubes	Propidium iodide
Flowcytometer (FACSCaliber)	Growth inhibitors
CO_2 -incubator	

Procedure:
All handling of PI should be done under protection from light!
Cells are incubated for the desired time, and cell suspension is transferred to polystyrene round bottom tubes. Propidium iodide is added, and after resuspension the cells are placed in the dark for 10 minutes. Then samples are analysed for red fluorescence (PI-staining), and the method is generally combined with forward and side scatter. 10 000 cells is analysed from each sample to get a statistically significant result.

3.5 Western blot analysis – analysis of protein expression

Western blot analysis is a method for detecting and quantifying specific proteins in a complex mixture of proteins. The protein samples are solubilised with detergents and reducing agents, denatured by boiling and separated electrophoretically on a polyacrylamide gel. After separation, the proteins are transferred to a nitrocellulose membrane and hybridised with antibodies that react specifically with antigenetic epitopes displayed by the target proteins attached to the membrane. This process is referred to as the Western blot. By adding a second antibody conjugated to horseradish peroxidase that catalyses the oxidation of luminol, the bound primary antibody can be detected by chemiluminescence and autoradiography.

3.5.1 Preparation of cell sample for electrophoresis

The cells are harvested and the proteins are solubilized by a lysis buffer. The purpose of this lysis is to solubelize all of the target antigens in a form that is immunoreactive and undegraded. Inhibitors of proteases are included in the buffer to prevent the release of intracellular proteases that could digest the target protein. It is important to keep the lysates on ice at all times to prevent protein degradation.

Equipment and solutions
Vortexer
PBS
RIPA buffer

RIPA buffer:

50 mM Tris pH 8, 0
150 mM NaCl
1% Triton
0, 5% NaDeoxycholate
0, 1% SDS
0, 5% Aprotinin
0,2 mM PMSF
10 µg/ml Leupeptin
5 mM NaF
10 mM β-glycerophosphate
1 Mm Na_3VO_4
In distilled water

The buffer is kept on ice when in use. It is stored at -20 ˚C without aprotinin.

PBS:

Solution A: 0,2 M NaH_2PO_4
31,2 g NaH_2PO_4 x $2H_2O$
Distilled water to 1 litre
Solution B: 0,2 M NaH_2PO_4
35,6 g NaH_2PO_4 x $2H_2O$
Distilled water to 1 litre
Solution A: 95ml
Solution B: 405 ml
NaCl: 81 g
Destilled water to 5 litres
Adjust pH to 7,4
Destilled water to 10 litres

Procedure:

Cells are collected by centrifugation and washed once with 400µl ice cold PBS. They are then resuspended in the appropriate amount of RIPA buffer (according to size of pellet), and incubated on ice for 20 minutes and votexed every 5 minutes. The cells are then centrifuged at 13000 rpm for 20 minutes at 4 ˚C to collect cell membranes and debris at the bottom of the tube. The supernatant is then transferred to a new tube, flash frozen in liquid N and stored
at -70˚C.

3.5.2 Determination of protein concentration

The Bio-Rad assay, based on the method of Bradford (Bradford 1976) is used to quantitate the concentration of protein in the cell lysates. The assay depends on the conversion of Coomassie brilliant blue G250 in dilute acid from brownish-orange to an intense blue colour when binding to protein. The absorbance maximum shifts from 465 nm to 595 nm and this is measured by using a spectrophotometer.

Equipment and solutions	
Eppendorf tubes	Distilled water
Bio-Rad protein assay	Disposable cuvettes (1, 5 ml)
γ- globulin	Spectrophotometer

Bio_rad protein assay:

Bio-Rad protein assay and distilled water is mixed in equal amounts.

γ- globulin:

γ- globulin (1 mg/ml) in distilled water.
While in use, the solution is kept on ice, and when not it use it is stored at - 20˚C.

Procedure:

A standard curve is made by dissolving 0, 5, 10, 15 and 20 µg of γ- globulin in distilled water to a total amount of 600 µl. The samples are prepared in parallels by adding 2, 5 µl of lysate to 600 µl distilled water. 400 µl Bio-Rad assay is added to all the standards and samples. The tubes are incubated for 10 minutes in room temperature before they are transferred to disposable cuvettes. The absorbance at 595 nm is measured according to the manual. The protein concentration in the lysates are then estimated from the obtained standard curve.

3.5.3 Separation of proteins by polyacrylamide gel-electrophoresis

SDS-PAGE (polyacrylamide gel electrophoresis) is a technique used to separate molecules on the basis of their charge and size by their different migration through a polyacrylamide gel in an electric field. Acrylamide is the basic element in the polyacrylamide gel. When ammonium persulfate (APS) is added, the free radicals generated initiate a chain reaction that results in the formation of long chains of acrylamide from the initial monomers. When N,N-methylenebisacrylamide (Bis) is added, the chains are cross bound and this results in a gel with pore size determined by the length of the chains and the degree of cross binding. The amount of Bis and acrylamide that is chosen depends on the size of the proteins under investigation and the desired degree of resolution. Before loading the proteins onto the gel, they are denatured and dissociated by boiling the samples for 5 minutes in the strongly anionic detergent, SDS, in combination with the reducing agent β-mercaptoethanol.
The denatured polypeptides bind SDS and become negatively charged.

Equipment and solutions	
Bio-Rad Mini Protean II gel apparatus	3 X SDS sample buffer
10 % SDS polyacrylamide gel	Heat block
Electrophoresis buffer	Syringe
Molecular weight standard	

10 % SDS polyacrylamide gel:

Separating gel:	**Stacking gel:**
3, 3 ml acrylamide	1, 3 ml acrylamide
4 ml dH2O	6, 1 ml dH2O
2, 5 ml 1. 5 M Tris-HCl, pH 8. 8	2, 5 ml o. 5 M Tris-HCl, pH 6. 8
100 µl 10 % SDS	100 µl 10 % SDS
50 µl APS	50 µl APS
5 µl TEMED	10 µl TEMED

3 X SDS sample buffer:	10 X Electrophoresis buffer:
1. 5 M Tris-HCl pH 6. 8	30 g/L Tris Base
10 % SDS	144 g/L Glycine
80 % Glycerol	10 g/L SDS
β-mercaptethanol	Dissolved in distilled water
dH2O	
Bromphenol blue	Diluted 1:10 when used

A small amount of bromphenol blue is added to the sample buffer.

Procedure:

Polyacrylamide gels are prepared according to the manual from Bio-Rad. For identification of p53, 10% separating gels are used. For preparation of samples, the lysate is mixed with 1/3 volume of 3 X SDS and with the addition of 1 X SDS to the volume of 30 µl. The samples are then boiled for 5 minutes and applied to the gel with a syringe. A molecular weight standard is also applied to the gel to visualize the migration of proteins of various sizes. The gel is run at 200 V, constant voltage, and the separation time depends on the size of the protein. For p53 analysis (53 kDa) the gel was run for approximately 45 minutes.

3.5.4 Transfer of proteins from gel to nitrocellulose membrane

Most Western blot analyses are carried out by direct electrophoretic transfer of proteins from the gel to a nitrocellulose membrane. The membranes bind proteins strongly and non-specifically. In these experiments a semi-dry transfer apparatus is used.

Equipment and solutions	**Blocking solution**
Platform shaker	Semi-dry transfer cell
Nitrocellulose membrane	Transfer buffer
Whatman 3MM paper	Ponceau solution

Transfer buffer:
48 mM Tris
39 mM Glycine
0, 0375 % SDS
20 % methanol
dH2O

Ponceau red solution:
0, 5 g Ponceau red
1 ml acidic acid
100 ml dH2O

Blocking solution:
8 % dried milk in TBS

TBS-Tween:
TBS with 0, 1 % Tween

TBS:
20 mM Tris
0, 8 % NaCl
Distilled water

pH is adjusted to 7, 6 with HCl.

Procedure:

When the proteins are separated on the SDS-polyacrylamide gel, the stacking gel is cut off, and the gel is put in transfer buffer to equilibrate. Six pieces of Whatman paper and one piece of nitrocellulose filter is cut at the exact size of the SDS-gel and soaked in the transfer buffer. 3 pieces of Whatman paper is placed in the transfer cell, the membrane is put on top of the papers, and the gel is put on top of the nitrocellulose membrane. Finally, the last 3 Whatman papers are placed on top of the gel. The gel and the membrane are therefore placed between the buffer-soaked filter paper, which serves as the ion reservoir and replaces the buffer tank. Air bubbles are then squeezed out by rolling a glass tube over the sandwich. It is important that the filter paper, membrane and gel are exactly aligned. The proteins are transferred from the gel to the membrane at 17 V for 25 minutes.

To visualize the amount of total protein in each lane as an extra control of equal loading, the membrane is incubated in ponceau for 2 minutes and washed with distilled water. The red dye binds to the proteins and thus indicates the amount of protein added to each lane. The colour is then washed away with TBS-Tween before further processing of the membrane. The sensitivity of the Western blot analysis depends on reducing the background of non-specific binding by blocking potential binding sites with irrelevant proteins. The membrane is incubated in a blocking solution of 8 % milk in TBS-Tween for 45 minutes in room temperature on the platform shaker, or in 4 ˚C over night with gentle agitation. Excess blocking solution is washed away with TBS-Tween.

3.5.5 Incubation of membrane with antibodies

The proteins on the nitrocellulose membrane are hybridised in two stages. First, the blot is incubated with an unlabeled antibody specific to the target protein in the presence of blocking solution. The blot is then washed with TBS-Tween and incubated with anti-immunoglobulin coupled to horseradish peroxidase (HRP). After further washing, the antigen-antibody complex is identified by chemiluminescence and autoradiography.

Equipment and solutions	TBS-Tween
Primary antibody solution	Rock-n-roller
Secondary antibody solution	Platform shaker

Primary antibody solution:
1 µg/µl primary antibody in 0, 5 % milk
0, 1 % sodium azid
Milk:Dried milk in TBS-Tween

Secondary antibody solution:
1:4000 secondary antibody coupled to horseradish peroxidase in 2% milk.

Procedure:
The membrane is incubated with the primary antibody solution for 1, 5 hours in room temperature on the rock-n-roller or over night at 4 ˚C with gentle agitation. The blot is then washed 1 x 10 minutes and 2 x 5 minutes in TBS-Tween on the platform shaker followed by incubation with the secondary antibody solution for 45 minutes at room temperature. The membrane is then washed 1 x 10 min and 3 x 5 minutes in TBS-Tween before visualisation by chemiluminescence and autoradiography.

3.5.6 Detection of proteins by chemiluminescence and autoradiography

The ECL Plus Western blotting detection system from Amersham Pharmacia Biotech provides a method for detection of immobilized antigens conjugated to HRP- labelled antibodies. HRP catalyses the oxidation of Lumigen PS-3 acridan substrate, generating thousands of acridinium ester intermediates per minute. These intermediates interact with peroxide under slight alkaline conditions to produce a sustained, high intensity chemiluminescence with maximum emission at 430 nm.

Equipment and solutions
Kodak X-omat 1000 processor
ECL plus Western blotting detection system
Hyperfilm™
Film cassette
Glad pack

ECL Plus Western blotting detection system:
Reagent A (ECL plus substrate solution containing Tris buffer)
Reagent B (Stock Arcidan solution in Dioxane and Ethanol)
Reagents A and B are mixed at a ration 1:40, with 2 ml reaction fluid for each membrane.

Procedure:
The excess TBS-Tween is drained off, and the membrane is placed with the protein side up on a piece of glad pack. The mixture of A and B is pipetted on to the membrane, and the blot is incubated for 5 minutes at room temperature. Excess detection fluid is drained off, and the membrane is placed in a plastic wrapping inside a film cassette with the protein side up. A sheet of hyperfilm is placed on top of the membrane in a dark room, and exposed for 1-30 seconds, depending on how strong the signal is. The exposed film is placed inside an automatic developer, and the antibody labelled protein will appear as a black line on the film.

4. RESULTS

4.1 The effect of EBV on cAMP-mediated inhibition of DNA damage-induced apoptosis

Previous work in our lab has shown that elevation of intracellular cAMP leads to G1 arrest in normal and malignant lymphoid cells (Blomhoff et al., 1987), and that cAMP also inhibits DNA replication and arrests the cells in S-phase (Naderi et al. 2005).

DNA damage responses are the basis for conventional cancer treatments like γ-irradiation and chemotherapy, and the p53 protein is a key mediator in this process. Our lab has recently demonstrated that elevation of intracellular cAMP also leads to inhibition of DNA damage-induced apoptosis, and results so far have shown that this inhibition is mediated by downregulation of p53 at the level of protein stabilisation (Hallan Naderi et al, manuscript in preparation).Recent experiments in out lab have shown that EBV infection of B-cells inhibits the antiproliferative effect of cAMP (Kloster et al., manuscript in preparation). We therefore wished to investigate whether EBV-infection of normal B-cells also would influence the cAMP-mediated inhibition of DNA damage-induced apoptosis.

To create EBV-transformed cells, we infected normal B-cells with Epstein-Barr virus. As a control for EBV-positive B-cells we used the lymphoid cell line Reh. These cells are EBV-negative cells that we for several years have used in our lab to study cAMP responses, and we have shown that the responses in Reh cells resemble those in normal B-cells. To increase intracellular levels of cAMP, we treated the cells with either forskolin or 8-CPT-cAMP prior to exposure to ionizing radiation. 8-CPT-cAMP is a membrane permeable analogue of cAMP, previously shown to activate protein kinase A, and forskolin is a deterpine compound isolated form plants, and it induces increased intracellular levels of cAMP by directly activating adenylyl cyclase (Seamon & Daly 1981).

4.1.1 EBV-cells are more resistant γ-irradiation

To induce DNA damage in the EBV-cells and Reh cells, we treated the cells with γ-irradiation. By exposing the cells to increasing doses of irradiation we wished to analyse the dose-response. Flow cytometric analysis was carried out 24 hour after exposure. We have assessed apoptosis by three different methods: 1) staining the cells with propidium iodide (PI), 2) scatter analysis and 3) assessing subG1 DNA content. By staining cells with PI it is possible to distinguish between dead and viable cells because PI is a DNA-binding dye taken up by necrotic and late apoptotic cells. Viable and early apoptotic cells exclude PI because they have an intact nuclei membrane. In Fig.13 panel A, a typical analysis of apoptosis by PI-staining is shown. Forward scatter light is related to cell size while side scattered light is an indicator of the cell's granularity. Apoptotic cells are smaller than viable cells, and more granular due to shrinkage of the cells, chromatin condensation, fragmentation of nuclei and formation of apoptotic bodies. Necrotic cells will be larger than viable cells due to swelling. Thus, by this method it is possible to separate cells into viable cells, apoptotic cells and necrotic cells. In Fig.13, panel B, a typical scatter analysis is shown.

The sub-G1 method is more apoptosis-specific and relies on the fact that after DNA fragmentation, there are small fragments of DNA that are able to be eluted following washing in either PBS or a specific phosphate-citrate buffer. This means that after staining with a quantitative DNA-binding dye, cells that have lost DNA will take up less stain and will appear to the left of the G1 peak. The advantage of this method is that it is very rapid, detects cumulative apoptosis and is applicable to all cell types. However in order to be detected in the subG1 area, the cell has had to loose a certain level of DNA, and also cells that have lost DNA for any other reason than apoptosis, will appear in the subG1 region. A typical subG1 analysis is shown in Fig.13, panel C.

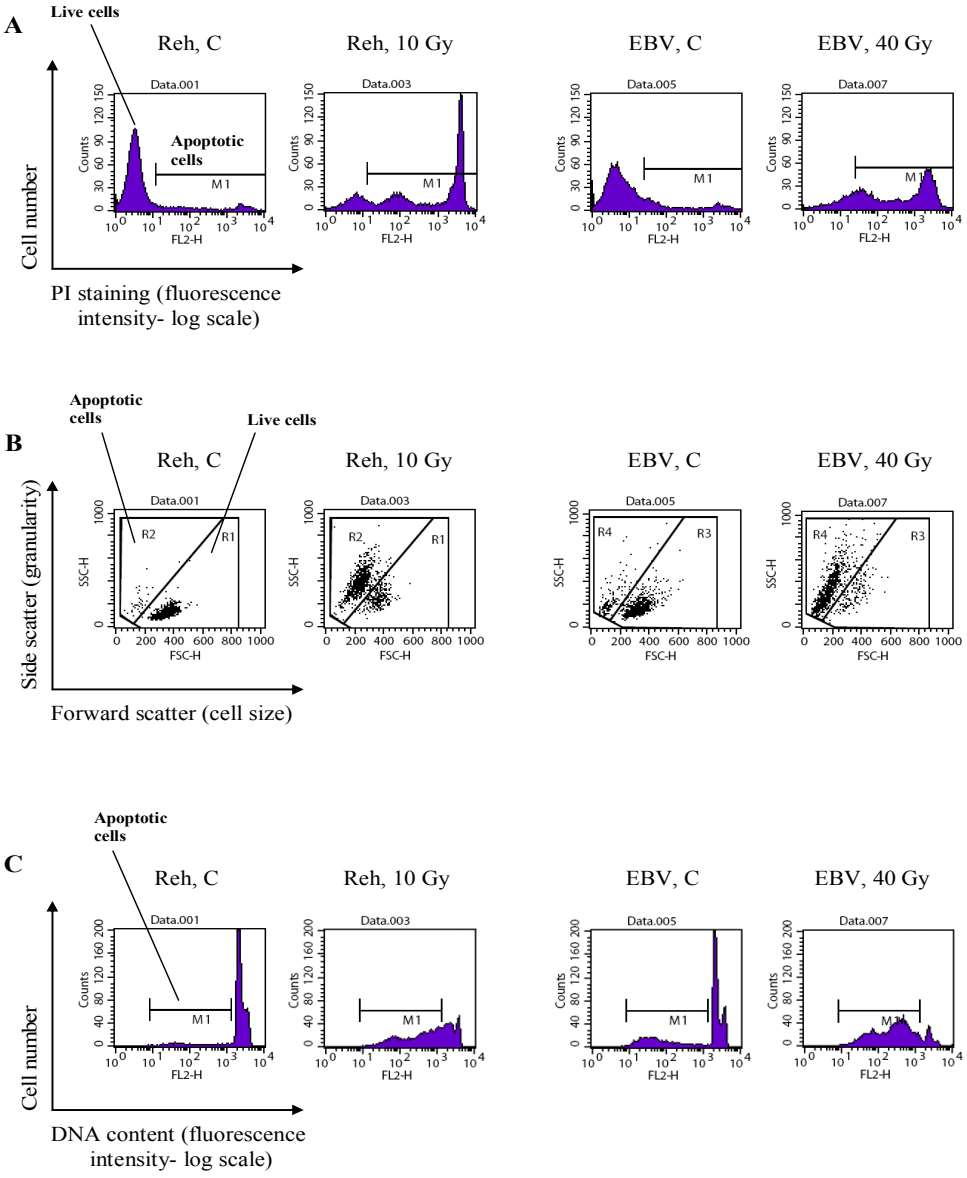


Figure 13: Analysis of apoptosis in Reh cells and EBV cells 24 hours after exposure of cells to γ-irradiation (Gy). Analysis of apoptosis is performed by flowcytometric analysis of cells stained with propidioum iodide (panel A), scatter profiles (panel B) or subG1 DNA content (panel C).

In order to induce apoptosis in cultures of Reh cells we typically irradiated the cells with 10Gy. As shown in Fig.14, we have induced apoptosis in approximately 70 % of the Reh cells 24 hours after exposure to this dosage of γ-irradiation. However, as shown in Fig.15, 10 Gy of irradiation had only minor effect on apoptosis of EBV-positive B-cells, indicating that EBV-positive B-cells are more resistant to irradiation-induced apoptosis than are EBV-negative B-cells. In order to induce similar extent of apoptosis in EBV-infected cells as in Reh cells, we had to use 40-60 Gy. As shown in Fig.16, 60 Gy induced almost the same level of apoptosis in EBV-cells as did 40 Gy, so we therefore routinely used 40 Gy in subsequent experiments with EBV-cells.

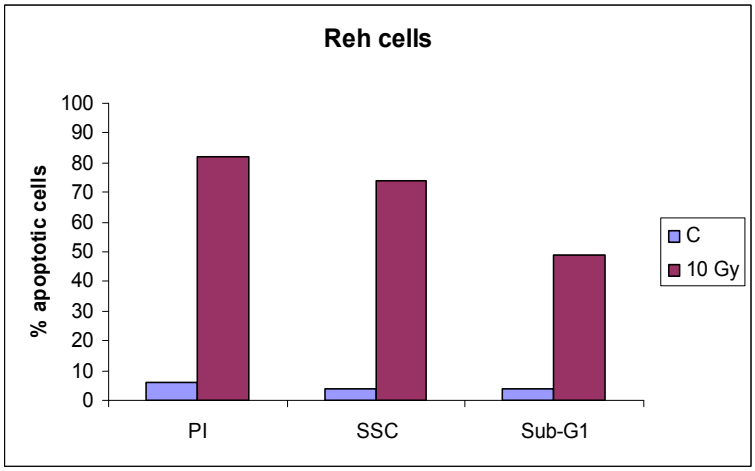


Figure 14: Reh cells were exposed to 10 Gy of γ-irradiation. Analysis was carried out 24 hours after exposure. The values are from one representative experiment out of three. PI indicates analysis of PI-staining, SSC indicates scatter analysis, and subG1 indicates analysis of cells with subG1 DNA content.

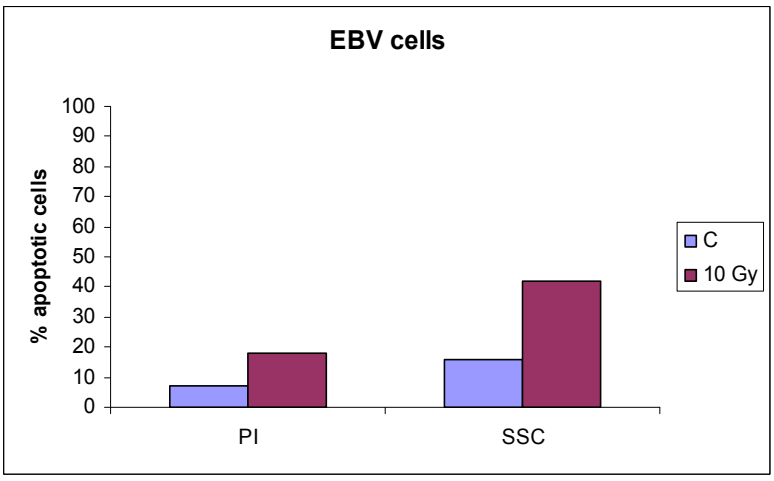
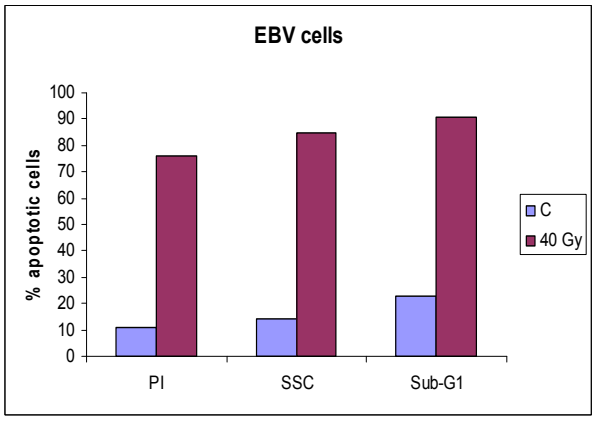


Figure 15: EBV-cells were treated with forskolin (60μM) for 45 minutes and then exposed to γ-irradiation of 10 Gy. Flow cytometric analysis was carried out after 24 hours. The values are from one representative experiment of three. PI indicates analysis of cells stained with propidium iodide, SSC indicates scatter analysis.



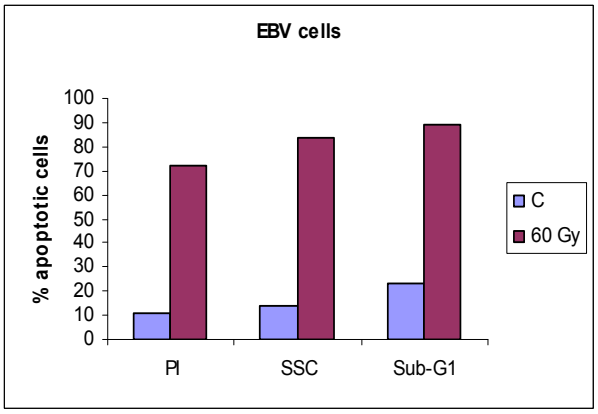


Figure 16: EBV-cells were exposed to γ-irradiation of 40 of 60 Gy. Flow cytometric analysis was carried out after 24 hours. The values are from one representative experiment of three. PI indicates analysis of cells stained with propidium iodide, SSC indicates scatter analysis, and subG1 indicates analysis of cells with subG1 DNA content.

4.1.2 EBV prevents the cAMP-mediated inhibition of DNA damage-induced apoptosis

In several papers from our lab during the last 10 years we have shown that cAMP inhibits proliferation of normal and malignant lymphoid cells, and we have recently also demonstrated that EBV infection of normal B-cell prevents this antiproliferative effect (Kloster et al., manuscript in preparation). Since we also recently have shown that cAMP inhibits DNA damage-induced apoptosis (Naderi et al, 2005), we wished to unravel whether EBV also would prevent the ability of cAMP to inhibit DNA damage-induced apoptosis.

The cells were treated with either 8-CPT-cAMP or forskolin before exposed to DNA damaging γ-irradiation. As shown on figure 17, analysis of cell death 24 hours after exposure to ionizing radiation showed that pre-treatment of Reh cells with forskolin or 8-CPT-cAMP prior to irradiation markedly inhibited the DNA damage-induced apoptosis. This is consistent with the observations that cAMP inhibits DNA damage-induced apoptosis in these cells. In EBV-cells, however, pre-treatment of the cells with forskolin or 8-CPT-cAMP prior to irradiation had only minor effect on the DNA damage-induced apoptosis (Fig.18).

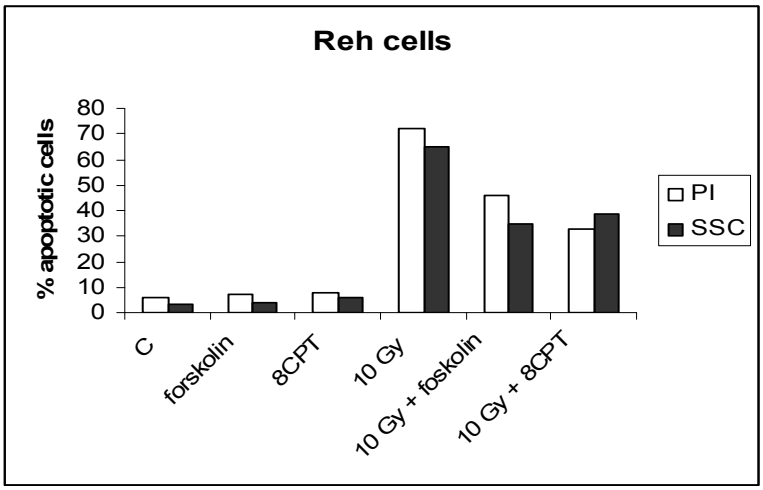


Figure 17: Reh-cells were treated with 8-CPT-cAMP (200μM) or forskolin (60μM) for 45 minutes before exposure to γ-irradiation. PI-staining (PI) and scatter analysis (SSC) were performed 24 hours after exposure. The values are from one representative experiment of three

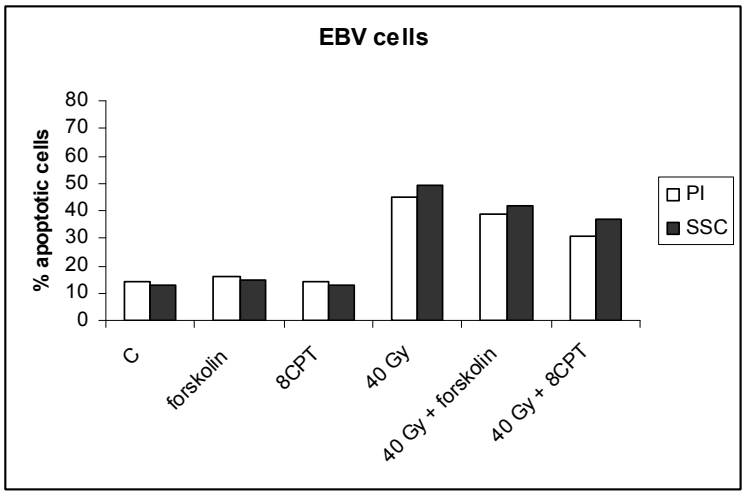


Figure 18: EBV-cells cells were treated with 8CPT (200μM) or forskolin (60μM) for 45 minutes before exposure to γ-irradiation. PI-staining (PI) - and scatter analysis (SSC) were performed 24 hours after exposure. The values are from one representative experiment of three.

4.1.3 Mechanisms for how EBV prevents cAMP-mediated inhibition of DNA damage-induced apoptosis.

After demonstrating that EBV indeed reduces cAMP-mediated inhibition of apoptosis induced by γ-irradiation, we wished to unravel the mechanisms involved. As indicated from recent results in our lab, cAMP inhibits DNA damage-induced apoptosis by down-regulation of p53 levels (Hallan Naderi et al., manuscript in preparation). To investigate the mechanisms whereby EBV renders the cells resistant to cAMP-mediated inhibition of apoptosis, we therefore investigated the effect of EBV on DNA damage checkpoints – including p53 levels. The cells were treated in the same manner as described in legends to figures 17 and 18, and the lysates were subjected to Western blot analysis of p53-levels and proteins involved in checkpoint-control.

As shown in Fig.19, γ-irradiation induced p53-stabilization as demonstrated by enhanced p53 levels and phosphorylation of p53 at Ser 15 in both Reh cells and in EBV-cells. In Reh cells, elevation of cAMP-levels by 8-CPT-cAMP markedly reduced this induced stabilization of p53, whereas in EBV-cells 8-CPT-cAMP was not able to reduce this stabilization. These results were in accordance with the inability of cAMP to prevent DNA damage-induced apoptosis in EBV-cells (see Fig.18).

In fig.20 are presented the similar results from forskolin-treated cells. Thus, again no inhibitory effect of cAMP was noted on p53 levels and stabilization of p53 (represented by enhanced phosphorylation of p53 at Ser 20) in EBV-cells, whereas both total levels of p53 and phosphorylation of p53 at Ser 20 were markedly reduced by forskolin in Reh cells.

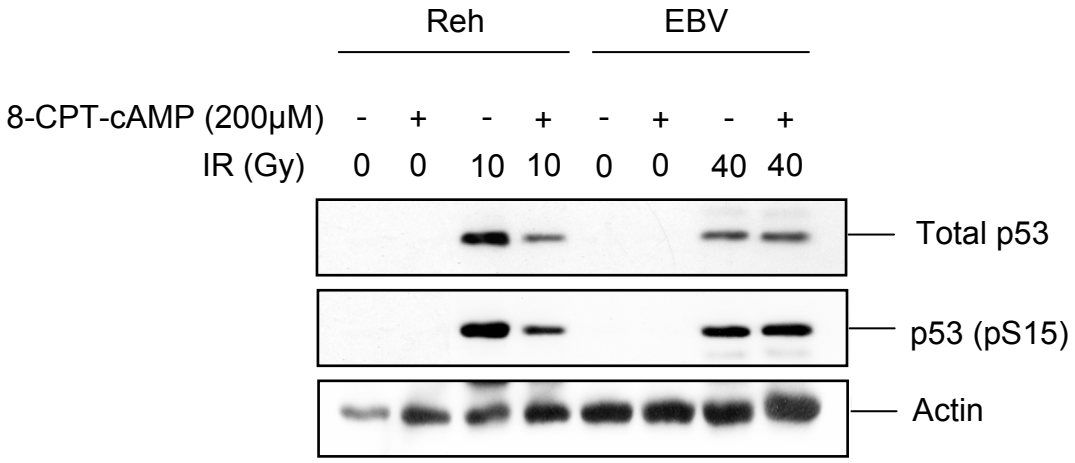


Figure 19: Effect of 8CPT-cAMP and γ-irradiation on p53 levels and p53 stabilization. EBV-cells and Reh cells were incubated with 8-CPT-cAMP (200μM) for 45 minutes prior to exposure to γ-irradiation. The cells were harvested 4 hours after irradiation, and equal amounts of protein (65μg) were separated on a 10% polyacrylamide gel before performing Western blot analysis using antibodies against p53 (total) and p53(S15). For visualization of loading the blot was rehybridized with antibodies against actin. One representative experiment of three is shown.

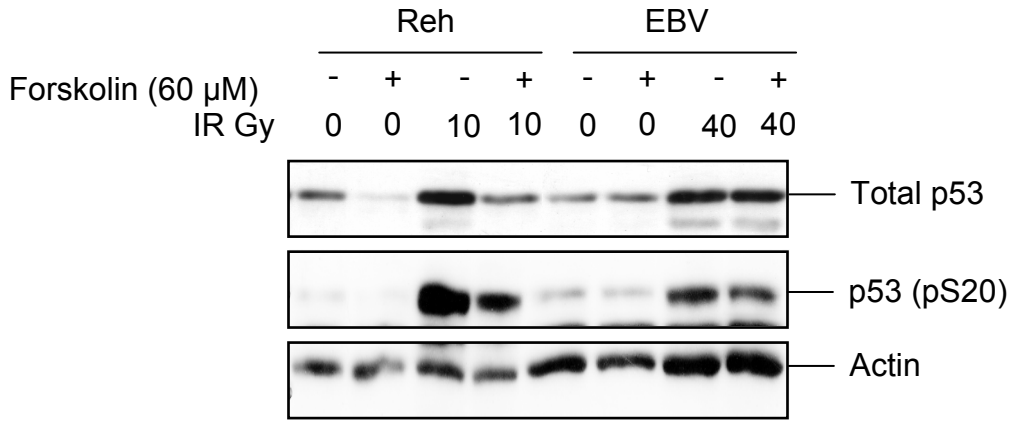


Figure 20: Effects of forskolin and γ-irradiation on p53 levels and p53 stabilization. EBV-cells and Reh cells were incubated with forskolin (60µM) for 45 minutes prior to exposure to γ-irradiation. The cells were harvested 4 hours after irradiation, and equal amounts of protein (65µg) were separated on a 10% polyacrylamide gel before performing Western blot analysis using antibodies against p53 (total) and p53 (S20). For visualization of loading the blot was rehybridized with antibodies against actin. One representative experiment of three is shown.

The upstream regulators of p53 are the checkpoint control proteins Chk1 and Chk2, both known to phosphorylate and thereby stabilize and activate p53. Active Chk1 and Chk2 are detected by antibodies against Chk1 phosphorylated at Ser317 or Ser345, or against Chk2 phosphorylated at Thr68, respectively. As shown in Fig.21, irradiation induced activation of both Chk1 and Chk2, but elevation of cAMP did not prevent this activation in either Reh cells or in EBV-cells. Thus, we could conclude that EBV-infection does not affect checkpoint-control upstream of p53, and cAMP-mediated inhibition of p53 levels is not a result of reduced activation of Chk1 or Chk2.

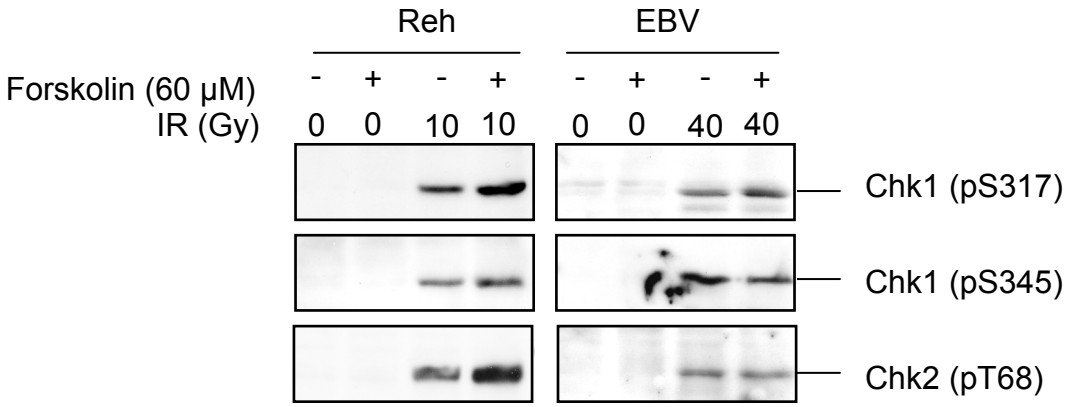


Figure 21: Effect of forskolin and γ-irradiation on DNA damage-induced checkpoint. Reh or EBV- cells were incubated with forskolin (60µM) for 45 minutes and exposed to γ-irradiation. The cells were harvested 4 hours after irradiation, and equal amounts of protein (65µg) were separated on a 10% polyacrylamide gel before performing Western blot analysis using antibodies against Chk1 (S317), Chk1 (S345) and Chk2 (T68). One representative experiment of three is shown.

5. DISCUSSION

5.1 EBV inhibits the effect of cAMP on DNA damage-mediated apoptosis

Epstein-Barr virus is a DNA tumour virus involved in development of certain B-cell and epithelial cell malignancies. Several years ago our lab showed that EBV-transformed B-cells were no longer inhibited by TGFβ (Blomhoff et al., 1987). Recently, we have shown that EBV infection of normal B-lymphocytes also inhibits the antiproliferative effect of cAMP in these cells, and it seems as though cAMP in EBV-transformed B-cells can no longer induce G1 arrest or inhibit S-phase progression (Kloster et al, manuscript in preparation).

The aims of this paper were to investigate whether EBV-infection of B-cells influences cAMP-mediated regulation of DNA damage-induced apoptosis triggered by γ-irradiation and, if so, to establish the mechanisms involved. As a control for EBV-positive B-cells we used the cell line Reh. Reh cells are EBV-negative cells that we for several years have used in our lab to study cAMP responses, and we have shown that the responses in Reh cells resemble those in normal B-cells. Thus, we have shown that cAMP leads to G1 arrest and inhibition of S-phase progression in Reh cells as well as in normal B-cells (Blomhoff et al., 1988; Naderi et al., 2005), and in both cell types the growth arrest includes downregulation of cyclin D3 (Naderi et al., 2000; Naderi et al., 2004) and Myc (Blomhoff et al., 1987). Taken together, we believe that Reh cells is a good model for EBV-negative B-cells to use as a control in our experiments.

Our experiments showed that EBV-transformed B-cells were more resistant to γ-irradiation, and needed larger doses than the Reh cell line to induce apoptosis. Further, we showed that EBV prevents the cAMP-mediated inhibition of apoptosis initiated by ionizing radiation. Results in our lab have shown that cAMP inhibits DNA damage induced apoptosis by downregulation of p53 at the level of protein stabilisation (Hallan Naderi et al., manuscript in preparation). To investigate the mechanisms whereby EBV render the cells resistant to cAMP-mediated inhibition of DNA damage-induced apoptosis, we therefore investigated the effect of EBV on the DNA damage checkpoint – including p53.

In this experiment, we showed that ionizing radiation activates p53 in both Reh and EBV-cells demonstrated by enhanced stabilisation of p53. Further, we showed that in Reh cells treated with forskolin or 8-CPT-cAMP and subsequently exposed to γ-irradiation, p53 stabilisation was inhibited. In EBV cells treated with the same agents there was little or no downregulation of p53, consistent with our results that EBV prevents the inhibitory effect of cAMP in response to DNA damage induced by ionizing radiation. To unravel whether EBV-transformation affected the checkpoint-control upstream of p53, we investigated the regulation of Chk1 and Chk2. We showed that activation of Chk1 and Chk2 were equally regulated in both Reh and EBV cells, suggesting that the inhibitory effect of EBV on cAMP-mediated regulation of DNA-damage-induced apoptosis is not mediated via checkpoint-control upstream of p53.

Work by O'Nions and Allday (2003) has shown that after exposure to the cytotoxic drug cisplatin, EBV disrupts the G1 checkpoint, but has no effect on p53 activation. Further, their work indicated that EBV acts downstream of p53 by blocking $p21^{(Cip1)}$ accumulation, leading to continuous activation of Cdk2, maintenance of pRB hyperphosphorylation and replication of DNA. In the same paper, they demonstrated that γ –irradiation could induce cell cycle arrest in EBV cells, and that this correlated with inhibition of Cdk2 activity. As reported by Cannel and coworkers (1996; 1998), EBV-positive cells that respond to cisplatin with reduced expression of $p21^{(Cip1)}$, respond to irradiation by increased levels of p53 and $p21^{(Cip1)}$. This indicates that cisplatin and γ –irradiation triggers different signals in EBV-infected cells, and that latency functions of EBV must discriminate between these. Recently, O'Nions and coworkers (2006), released further results showing that when proliferating B-lymphocytes respond to γ –irradiation, $p21^{(Cip1)}$ protein accumulates and the cells undergo cell cycle arrest irrespective of whether EBV is present or not, and that the phosphorylation, stabilization and activation of p53 appear to be unaffected by EBV. This correlates with our results that EBV does not act upstream of p53 in response to double stranded DNA damage.

In the same publication by O'Nions and coworkes (2006) it was shown that EBV cells tend to accumulate in the G1 and G2 phases of the cell cycle when treated with ionizing radiation. In our experiments, however, the lymphocytes respond to γ –irradiation mainly by apoptosis. This could be related to the dosage used. O'Nions and coworkers used a dosage of 8, 5 Gy in their experiments, whereas in our work we used 40 Gy to induce DNA damage-induced apoptosis.

The mechanism behind the increased resistance to DNA damage-induced apoptosis in EBV cells is not fully known. In 1998, Komano and coworkers showed that EBV-positive cells were more resistant to apoptosis than were EBV-negative cells, and that EBV-infected cells expressed the Bcl-2 protein through which cells might become resistant to apoptosis. This was the first report that Burkitt's lymphoma type EBV-infection confers apoptosis resistance even in the absence of LMP1 or BHRF1 – both which are known to have an antiapoptotic function. The EBV protein BHRF1(Bam HI fragment H rightward open reading frame 1) shares a distant collinear amino acid sequence homology with the protein coded by the Bcl-2 protooncogene, and can protect against apoptosis induced by the DNA damaging agents cisplatin, etoposide and mitomycin C (Tarodi et al., 1994). It has been shown that transfection with the EBV genes LMP1 or EBNA2 upregulates Bcl-2 in B-cell lines, indicating a role of this anti-apoptotic protein in EBV-protection against apoptosis. In fact, Roth and coworkers (1996) have shown that LMP expression also provides resistance against hydrocortisone-induced apoptosis, and that this resistance possible is due to upregulation of Bcl-2.

Another study shows that EBV-negative Burkitt lymphoma-derived cell line, BL41 are extremely sensitive to genotoxic drugs, and rapidly undergo apoptosis, whereas latent infection with EBV protects these cells from both apoptosis and cell cycle arrest (Wade and Allday, 2000). Interestingly, it seemed as though LMP1 was not involved in this protection, and that levels of Bcl-2 and related proteins did not change significantly during apoptosis, nor was the level of the anti- or proapoptotic proteins predictive of the outcome of the treatment .Recently, Clybouw and coworkes (2005) showed that EBV infection leads to post-transcriptional downregulation of expression of the proapoptotic protein Bim, and that EBV-mediated resistance to growth factor deprivation in human B-lymphocytes is dependent on BimEL expression, indicating a contribution to the oncogenic potential of EBV.

It would be interesting to investigate which EBV-genes inhibit the cAMP-mediated response. LMP1 is shown to be important in EBV-mediated B-cell proliferation and mimics an activated CD40 receptor, and thereby the CD40/CD40L interaction between B-cells and other cells in the immune system (Uchida et al., 1999; Lam and Sugden, 2002; Panagopopoulos el al., 2004). However, we have shown in our lab that CD40/IL-4 mediated regulation of normal B-cells do not counteract cAMP-mediated growth inhibition (Kloster et al., manuscript in preparation).

The mechanisms by which EBV inhibits the effects of cAMP on growth arrest and apoptosis are currently a subject of investigation in our lab. The level of inhibition could theoretically be upstream of p53 in the DNA damage pathway, in the synthesis or function of cAMP or in the cAMP pathway. Recent experiments in our lab indicate that EBV-infection of B-cells inhibits adenylyl cyclase activation and cAMP accumulation (Kloster et al., manuscript in preparation), and this fits with our results demonstrating that EBV-infection renders the B-cells irresponsive to the cAMP-mediated destabilization of p53, and that it does not affect the checkpoint-control upstream of p53.

5.2 Clinical implications of our results

Normal cell growth is dependent on a fine tuned balance between positive and negative growth signals, and a balance between cell proliferation and programmed cell death. Any changes in these processes may lead to cancer development, and the disruption of DNA damage checkpoint (ATM-Chk2-p53) and/or the intrinsic pathway of the apoptosis machinery are both very common in cancer cells (Shin et al., 2006; Soung et al., 2006; Ishikawa et al., 2006). Cancer rises as a result of a multistep accumulation of genetic changes that can occur in tumour suppressor genes and/or oncogenes. These changes do not only facilitate tumour formation, but can also influence the outcome of cancer therapy.

DNA damage responses are the basis for conventional cancer treatments like γ-irradiation and chemotherapy, and the p53 protein is a key mediator of the ATM-dependent DNA damage response cascade following cellular exposure to ionizing radiation (for a review, see Cuddihy and Bristow, 2004; Choudhury et al., 2006). By inducing DNA damage in the cancer cells, the cells are either marked for apoptosis or arrested at different stages in the cell cycle. The general response of lymphoid cells to DNA damage is apoptosis. A major problem with conventional cancer therapy is that usually only a subgroup of patients responds to the treatment given. In addition, side effects of cancer treatment limit the dose-efficiency of the treatment. In future research and development of new cancer treatments, the aim must be to create combinational regimes with higher efficacy and lower dose requirements so that more patients will respond with fewer side effects.

Many tumours express high levels of cAMP or cox-2 (induces PGE2-mediated induction of cAMP) (Gunnarson et al., 2006). Previously we have shown in our lab that elevation of intracellular cAMP leads to G1 arrest in normal and malignant lymphoid cells (Blomhoff et al., 1987). We have recently demonstrated that elevation of intracellular levels of cAMP also inhibits DNA replication and arrests the cells in S-phase (Naderi et al., 2005). A number of current cancer therapy regimes rely on the use of drugs whose cytotoxic activity depend on ongoing DNA replication. Elevation of intracellular cAMP prior to irradiation or chemotherapy has recently been shown to inhibit DNA damage induced-apoptosis (Naderi et al., 2005), rendering tumours expressing high levels of cAMP resistant to this antitumour treatment. This leads to the possibility of using cAMP antagonists/PKA inhibitors or cox2-inhibitors prior to conventional therapy to increase the effect of γ-irradiation or chemotherapy in tumours expressing high cAMP levels and thereby improving the dose-efficiency of the treatment. This is at present the subject for further research in our lab. Of note is that a recent paper demonstrated induced apoptosis of neuroblastoma cells treated with a combination of cox-2 inhibitors and low doses of the cytotoxic agents doxorubicin or etoposide (Lau et al., 2006). The authors did not, however, relate the cox-2 inhibition to inhibition of cAMP levels.

Epstein-Barr virus is a DNA tumour virus belonging to the γ-herpes virus family. More than 90% of the adult population is seoropositive for EBV as a result of an asymptomatic primary infection in the childhood. Like all herpes viruses, EBV is able to persist in the host for life, but in the majority of healthy carriers the virus causes no disease. This is because a delicate balance between the host immune system and the virus is maintained. Acute EBV-infection in the adult can cause infectious mononucleosis (IM), characterized by the triad of fever, lymphadenopathy and pharyngitis (Papesch & Watkins, 2001). EBV is also shown to cause different types of cancer and proliferative disorders including Burkitt's lymphoma (Epstein et al., 1964), nasopharyngeal carcinoma (Henle & Henle, 1976), gastric cancer (Osato & Imai, 1996), Hodgkin's lymphoma (Deacon et al, 1993), post-transplant lymphoproliferative diseases (Swinnen, L.J., 1999) and AIDS-associated lymphomas.

We have shown that cAMP inhibits DNA damage induced-apoptosis and that EBV prevents this inhibition, rendering the cells resistant to cAMP inhibition. In clinical practice, this means that patients with EBV-positive malignancies may not benefit from a combinational treatment of PKA inhibitors and irradiation/chemotherapy as postulated for normal B-cells - even in tumours expressing high levels of cAMP. Because the antiproliferative effect of cAMP is neutralized in EBV-cells, one would expect that EBV-positive cancers are more susceptible to cytotoxic agents. However, this is shown by ourselves and by others (Clybouw et al., 2005) not to be the case. In fact, we have shown that EBV-cells are more resistant than EBV-negative cells to DNA damage induced-apoptosis. This may indicate that patients with EBV-positive malignancies need higher doses of treatment to respond to conventional treatment. And, as EBV not only infects B-cells, but also T-cells and epithelial cells, these results may have implications in other types of cancers as well.

EBV has recently been shown in our lab to exert its inhibitory effect on cAMP-mediated G1-arrest at the level of adenylyl cyclase (Kloster et al., manuscript in preparation), and further unravelling the mechanisms by which EBV brings out its inhibitory effects on the ability of cAMP to prevent apoptosis in response to DNA damage, is a promising area of further research in our lab. Hopefully this research will provide us with some novel tools to improve antitumour treatment of EBV-positive malignancies in the future.

5.3 Conclusions

Our aims for the current project were to unravel whether EBV-infection of normal B-cells prevents the cAMP-mediated inhibition of DNA damage-induced apoptosis in response to γ-irradiation, and if so, to establish the mechanisms involved.

We could conclude that:

1) EBV-transformed B-cells are more resistant to DNA damage-induced apoptosis, and that higher doses of γ-irradiation are required to trigger an apoptotic response.

2) EBV inhibits the cAMP-mediated regulation of DNA damage-induced apoptosis triggered by γ-irradiation.

3) The mechanism by which EBV regulates cAMP inhibition of apoptosis when exposed to ionizing radiation does not seem to involve the DNA damage checkpoint- control upstream of p53 but rather affect the cAMP-mediated pathways as such. Thus, EBV-infection prevents cAMP-mediated downregulation of p53 without affecting activation of Chk1 or Chk2.

6. Reference list

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